ASPECTS OF THE ECOLOGY AND PHYSIOLOGY OF THE DIGENETIC TREMATODE, PROCTOCELES SUBTENUIS (LINTON)

By

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Prootoeoes subtenuis (Linton) a digenetic trematode that had previously only been found as a parasite in the hind gut of sparid and labrid fish, was first described as a parasite of the lamellibranch Scrobicularia plana (da Costa), in the Thames Estuary, by Freeman and Llewellyn (1958).

During the present investigation Prootoeoes was only found within S. plana collected from localities along the north coast of the Thames Estuary, although the lamellibranch host was found to be common in neighbouring areas. An investigation of the S. plana from eight locations along the north coast revealed that the abundance of the parasite was far from uniform; the S. plana collected from certain localities being heavily infected whereas those collected from localities a short distance away (1 mile or less) were often only rarely infected. The pattern was repeated in each of the three years of study.

The investigation of a heavily infected population of S. plana over the period of study demonstrated that the parasite was very successful. From a level of infection of 2 – 3 Prootoeoes per host in 1967 an increase occurred to a level of infection in 1969/70 at which over 95% of all S. plana collected were infected and with an average of 4 – 5 Prootoeoes
per host. As many as 14 Prootoeces were recovered from a single host and the number of Prootoeces per Scrobicularia increased pari passu with the size of the host.

The discovery that the pattern of distribution of Mytilus edulis (L) was very similar to that of Prootoeces suggested that this lamellibranch could be involved in the life cycle of Prootoeces in the Thames Estuary. This was further suggested by the discovery of sporocysts from M. edulis, collected from two areas where Prootoeces was common, that closely resembled those described for members of the genus by American workers. On the basis of the evidence a life cycle has been suggested and some general topics considered.

The availability of large numbers of Prootoeces allowed for an investigation of some aspects of the physiology of this animal. In order to do this satisfactorily the environment of Prootoeces, the kidney of S. plana, was also investigated.

Prootoeces and the kidney fluid of S. plana were found to be isosmotic with respect to the external medium from a depression of freezing-point of about 0.5°C to 2.0°C. Prootoeces was found to be able to survive for significant periods in sea water dilutions of between 20% and 100%; the greatest duration of survival being in 30% and 50%
sea water. The significance of this latter observation is uncertain although it has been suggested that this osmotic concentration could be similar to that occurring in the hind gut of a fish.

The oxygen tensions within the kidney of *S. plana* have been shown to fall, during a period of exersion, to levels approaching zero, but no evidence has been obtained that the haemoglobin of *Protoeces* would dissociate its oxygen under such conditions. Observations of the behaviour of the pigment in vivo and its possible functions have been discussed.
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INTRODUCTION

Adult digenetic trematodes are typically parasites of vertebrates. Freeman and Llewellyn (1958) described sexually mature, unencysted digenocans from the mud-dwelling lamellibranch, Scrubicularia plana (da Costa), collected from Chalkwell in the Thames Estuary.

The trematodes were identified as belonging to the genus Prootoeces (Odhner, 1911) and of the family Fellodistomatidae, sub-family Haplooladinace (Odhner, 1911).

The genus Prootoeces was established by Odhner (1911) for Distomum maculatum (Locca, 1901) and Prootoeces erythraeus (Odhner, 1911). Since that date many new species have been described from fish and the occurrence of Prootoeces from invertebrates from many parts of the world has been noted on eight occasions. Unfortunately, the characteristics of many of these species are based upon those observed in only a few specimens collected from fish. The ability to compare large numbers of Prootoeces from a single species of invertebrate host has demonstrated the extent of intraspecific variation that is possible within this genus, and thus the validity of some of the species has been questioned.

An excellent example of this is the case of Prootoeces major (Yamaguti, 1934). This was a new species described from the fish,
Pogrosomus auratus from Japan. One of the distinguishing features of this species was the existence of a tri-lobed ovary. Freeman (1962) suggested on the basis of finding a tri-lobed ovary in a specimen of P. subtenuis from S. plana, that P. major must be considered synonymous with P. subtenuis. Dollfus (1964) in describing P. progeneticus from the top-shell Gibbula sp. stated that the shape of the ovary could not be considered as a diagnostic feature as specimens examined by him had spherical, oval or tri-lobed ovaries.

Similarly Ichihara (1965) in describing Proctoeces from the top shell Turbo (Batillaria) cornutus and Dolgyok (1967), in describing a species of Proctoeces from the prosobranch, Rissoa splendidia, mentioned that the shape of the ovary was varied, being oval, globular or tri-lobate.

Thus it would seem to be strongly suggested that the shape of the ovary is a poor diagnostic feature. The question of what characteristics can be accepted as diagnostic has been discussed in detail by Dollfus (1964).

The systematics of the genus Proctoeces thus presents many problems, which are made more difficult by the fact that authors are not in agreement with their synonyms. It is not intended within the
confines of this thesis to discuss the specific identification of members of the genus as this has been dealt with, in detail, in the literature. The specific status of the species *maculatus*, *erythraeus*, *subtenuis*, *insolitus*, *ostrae*, *major*, *magnorae* and *macrovitellus* has been discussed in detail by Freeman and Llewellyn (1958). This discussion has been continued in the literature and further points raised by many authors, including Stunkard and Uzmann (1959), Manter and Pritchard (1962), Freeman (1962a), Dollfus (1964) and Loos-Frank (1969).

Freeman and Llewellyn (1958) considered that the features exhibited by the *Proctoeces* discovered from *S. plana* in the Thames Estuary were consistent with the description for *Proctoeces subtenuis* (Linton, 1907), Hanson, 1950, a species which had only been recorded up to that date as a parasite of the hind gut of marine fish belonging to the families Labridae and Sparidae, from the Red Sea, New Zealand and the Eastern Seaboard of America.

Loos-Frank (1969) discovered a species of *Proctoeces* from *S. plana* on the German coast that she preferred to name *P. sorobiculariae*. She appears to base this renaming mainly upon the belief that significant zoogeographical barriers, such as the
Atlantic Ocean, ought to be insuperable barriers for fish as well
as littoral molluscs, and thus the finding of the same parasites
in Europe and in Bermuda would be unlikely. What she has not
considered is the fact that many littoral molluscs are transported
from continent to continent as fouling organisms on ships
(see page 120) or even accidentally introduced along with other
animals (see page 82).

Until specific material can be compared from various
localities there does not seem to be any reason not to regard the
specimens of Proctoeces from S. plana in Europe as P. subtenuis,
and accept that some species of Proctoeces have wide
distributions. This would apply especially to P. maculatus, a
species that, since its original discovery by Looss (1901), has
been described from fish and from molluscs from many parts of the
world, and is considered by some authors (Stunkard and Uzmann, 1959;
and Manter and Pritchard, 1962) as synonymous with P. subtenuis.

Description of Proctoeces from Scoobicularia plana.

For an excellent, full account of the functional morphology of
this digenean, the reader is referred to the work of Freeman and
Llewellyn (1958). The main features are summarised below and
accompanied by drawings and photographs of stained, lightly
squashed specimens, (Figs. 1 and 2).

Upon removal from the host the Proctoocoes is highly active with
most elongation and contraction occurring in the region anterior to the
ventral sucker. Possibly the most immediately noticeable feature of
these removed trematodes is that they are red or pink in colour. This
colouration is generally distributed in the tissues of the animal,
with the intensity of the colour varying with the size of the
Proctoocoes. Freeman and Llewellyn (1958) demonstrated that this colour
was due to a native haem pigment.

Although this colouration is so noticeable in Proctoocoes collected
from S. plana from Chalkwell, it has not been recorded as occurring in
Proctoocoes recovered from fish or from other invertebrates. This
includes Proctoocoes obtained from S. plana from Germany and described by
Loos-Frank (1969). This author makes no comment about colouration of
the worms.

Proctoocoes from S. plana from Chalkwell can attain a maximum length
(when gently squashed) of about 4.8 mm. They are generally cylindrical,
tapering at each end, with the ventral sucker situated at about one
third of the body length from the anterior end, and the integument is without spines.

The mouth is sub-terminal and is succeeded by the pre-pharynx, which in turn communicates with the pharynx and then leads into the oesophagus. All these three anterior regions of the alimentary canal are lined with epidermis but not so the succeeding two simple intestinal caeca which almost reach the posterior end of the body.

The uterus in sexually mature specimens is always full of eggs and with the genital pore opening between the oral and the ventral sucker, the exact position relative to the suckers varying with the state of elongation or contraction.

The testes are diagonally tandem, situated posterior to the single ovary. The vitellarium is poorly developed, situated mainly in the peripheral regions of the body, the extent varying in different individuals.

The excretory system is extremely prominent with a Y-shaped bladder, the paired arms of which extend as far forwards as the posterior limit of the pharynx. Posteriorly at about the level of the testes, these arms unite to form the median stem that runs backwards as a relatively wide tube.
The unusual availability of large numbers of a digenean makes Protoeces an ideal subject for morphological and physiological study.

Part of this thesis is concerned with aspects of the physiology of Protoeces. This is a field of study that has often been neglected in the case of digeneans. This is possibly due, in many instances, to lack of material and often because of the technical difficulties imposed by the small size of most trematodes. In the present case, even with the availability of large numbers of trematodes, the small size of the animal caused technical difficulties. This necessitated a study of techniques and so part of the thesis is concerned with this aspect of the work.

A parasite, as with any animal, must be studied with respect to its environment. As many aspects of the physiology of E. plana, and for that matter lamellibranchs in general, have not been previously investigated in any detail, much of this thesis concerns work that has been carried out upon the host of Protoeces.

The fact that the host is an invertebrate makes the study even more interesting as it provides a set of conditions that can be compared with those that the parasite would probably find in the vertebrate host. However, comparisons of this nature must be viewed with care as
differences or similarities that may appear significant to the
investigator may be of little importance to the trematode.

The Host: Scrobicularia plana (da Costa)

*Scrobicularia plana* is a lamellibranch belonging to the family
Scrobiculariidae of the super-family Tellinacea. It is the only
British species of the genus, but it has, in the past, been known by
a number of synonyms under twelve different genera (see Forbes and
Hanley (1953) and Clay (1962) for a list of the synonyms). It is a
mud-dwelling lamellibranch found around European coasts from
Scandinavia, into the Mediterranean and as far south as Senegal. It
is common on British shores, being characteristic of estuaries of the
south and east of England but it is scarce and local in the north
(Spooner and Moore, 1940).

It can usually be found over a wide area of the intertidal zone,
in the region of mid-tide level and according to Tebble (1966) can
attain a maximum length of about 6.35 cm.

The animal lives in a permanent burrow, which varies in depth
according to the nature of the substrate and the size of the animal.
It is usually found at a depth in the region of six inches below the
surface but it may be as much as ten inches or as little as one inch.
This means that the animal lives in the black, sulphide layer of the mud. Of interest is the fact that the mud actually surrounding the animal is light in colour, and therefore oxidised.

The animal lies towards the vertical in the burrow, maintaining contact with the surface of the mud by its long, highly extensible, siphons (see Fig. 3). The animal is a deposit feeder, sucking up the mud and microorganisms via the inhalant siphon.

Hughes (1969) has shown that surface deposit feeding may occur throughout the period of low tide if the surface water remains. During immersion deposit feeding may be limited to the mouth and sides of the burrow, possibly in order to conceal the siphon tips from predators. Hughes (1969) has also shown that although S. plana is primarily a deposit feeder it may obtain some of its food by suspension feeding.

The internal structure of S. plana is illustrated in Fig. 4. Proctoeceae occurs in the kidney region which, as in most lamellibranchs, consists essentially of a proximal glandular, ciliated tubule (the organ of Bojanus) which drains the pericardium through the renopericardial opening and a ureter which conveys the excretory products to the suprabranchial space. From here they are conveyed to the exterior via the exhalant siphon (Potts and Parry, 1964).
Fig. 3. Typical attitude of *Scrobicularia plana* in burrow. (Natural size).
Fig. 4. *Scrobicularia plana*, right valve removed (X 2)

(Adapted from Yonge, 1943.)

- AA: Anterior adductor muscle
- DG: Digestive gland
- ES: Exhalant siphon
- F: Foot
- G: Gill
- H: Heart
- IS: Inhalant siphon
- K: Kidney
- P: Palp
- PA: Posterior adductor muscle
S. plana is not collected in Britain for food and it is not popular as a bait for fishing. To the author's knowledge, there are no records in the literature of whole Sorobicularia plana being eaten by fish. Clay (1962) states that Hardman (1893) and Scott (1895) have found S. plana in the stomachs of Plaice and Cod. Careful reading of the original references shows that the authors refer to Sorobicularia alba, which is another member of the family Sorobiculariidae; now known as Abra alba (W. Wood).

Whether it forms part of the diet of birds that feed in the intertidal zone has not been ascertained, although the author has never witnessed such an occurrence in the Thames Estuary.

It would seem, as commented by Freeman and Llewellyn (1958) that the nature of its habitat, occurring as it does well below the surface of the mud, would make it relatively inaccessible to most predators.

It is known that very low temperatures may have a detrimental effect upon Sorobicularia beds. Stopford (1951) noted a considerable reduction in the numbers of the mollusc in the estuary of the River Dee in Cheshire, after the winter of 1946. Similar extensive mortalities of S. plana were observed by Newell (1964b) to have occurred in and around the Thames Estuary as a result of the severe winter of 1962/3.
Prisman (1963) reported the virtual elimination of _S. plana_ from the vicinity of Chalkwell, in the Thames Estuary, where it acted as the host for *Protoeces*.

Evidence of extensive mortalities at some point in time was provided in many areas by the existence of very large numbers of empty shells. Whether these were solely due to the 1962/3 winter cannot be ascertained but at most of these localities only a very few living Scrobiculaaria could be found, and at some the lamellibranch was absent.

The finding, in 1967, that Scrobiculaaria were still parasitised by *Protoeces* provided the opportunity for an interesting study of a sexually mature digenean from an invertebrate host. If one accepts the contention of Wright (1960) that there can be little doubt that the flukes were originally parasites of molluscs, then this occurrence provides a rare opportunity to study conditions, similar to those that might have been experienced by some of the early flukes. This is not to say that this condition in *Protoeces* is necessarily of a primitive nature, but only that it provides an interesting comparison with the conditions encountered by adult *Protoeces* in vertebrates.
The thesis is divided into two main sections: The first section discusses the ecology of the parasite in the Thames Estuary and the surrounding area. The results of the ecological survey prompted an investigation into the life history of Proctocephalus in the Thames Estuary. This is discussed and the probable life cycle in this area is outlined. On the basis of this suggested life cycle certain points are discussed. As previously stated (P 12) a discussion of the systematics of the genus Proctocephalus is avoided.

The phenomenon of progenesis is briefly discussed with respect to Proctocephalus as a parasite of S. plana.

The second section of the thesis discusses the results of investigations into aspects of the physiology of both the parasite and the host. The extent of salinity fluctuations and the osmotic responses of both parasite and host have been examined.

The existence of a native haem pigment in Proctocephalus prompted an investigation into its properties in relation to the oxygen tensions prevailing in the kidney of S. plana. This latter study necessitated the use of a miniature polarographic electrode to measure internal oxygen tensions and an oxygen monitor system to determine whole animal respiration.
There is a surprising lack of data in the literature on aspects of the physiology of lamellibranchs and especially of trematodes. It is hoped that some contribution has been made as a result of the present investigation.
SECTION I
Chapter I

GENERAL TECHNIQUES

The techniques discussed in this chapter are those that were used throughout the investigation and will not be discussed further in subsequent chapters.

1. Opening of the Scrobicularia and obtaining the Proctoeoes

This was accomplished very simply. The cutting of the anterior and posterior adductor muscles (see Fig. 4) caused one valve to lift up under the influence of the elastic ligament. It was then a simple operation to completely remove one valve and thus expose the kidney region of the lamellibranch.

An alternative method was to tap the bivalve sharply on a solid surface. This usually caused a break in one valve along the line of one of the early growth rings. The subsequent lifting of the pieces of the shell due to the action of the ligament was usually sufficient to expose the kidney region.

Once the kidney was exposed, its tissue could be eased apart whilst being viewed through the low power objective of a binocular microscope (x4 magnification). Large Proctoeoes were easily visible owing to their pink or red colouration whereas care had to be taken
to ensure that the small, transparent trematodes were not 'lost' within the glandular tissue of the kidney. These usually exposed themselves to the investigator by their extensive movements.

The Protooeoa were pipetted out of the kidney, placed in 100% sea water and were stored at $6^\circ C \pm 1^\circ C$ for the short time before use.

2. Ageing the Sorobicularia

When needed, an estimate of the age of a Sorobicularia was obtained by comparing the length of the shell with the age/length data compiled by Green (1957). Although this information was calculated for a population of *S. plana* in the Gwendraeth Estuary in South Wales it was found to apply to the population of *S. plana* in the Thames Estuary. Estimates of the age calculated using the information of Green (1957) corresponded well to the age ascertained by counting the annual growth rings on shells on which they were clearly visible. "Homologising the rings" on a shell with those on larger and smaller shells (as suggested by Green, 1957) provided a check when further accuracy was needed.

3. Pasteurisation of sea water

Sea water for use in experiments was prepared by initially filtering it through a milliport filter ($0.22 \mu$ pore diameter). Pasteurisation was accomplished by heating it up to $60^\circ C$, three times.
Between each heating the sea water was allowed to cool to room temperature. Once Pasteurised the sea water was placed in a stoppered bottle and stored in a refrigerator at 6°C ± 1°C. Before use care was taken to ensure that it was fully aerated.

4. Statistical techniques

a. Calculation of standard deviation and standard error of the mean (Hale, 1965).

\[
S.D. = \sqrt{\frac{1}{n-1} \left( \frac{\sum x^2}{n} - \left( \frac{\sum x}{n} \right)^2 \right)}
\]

\[
S.E.M. = \frac{S.D.}{\sqrt{n}}
\]

b. Test for difference between two sample means (Moore and Edwards, 1965).

\[
U = \frac{x_1 - x_2}{\sqrt{\frac{S.D._1^2}{n_1} + \frac{S.D._2^2}{n_2}}}
\]

To test whether \( \bar{x}_1 \) is significantly different from \( \bar{x}_2 \), compare the obtained value of u against tables of u.
c. Regression lines were calculated by the method of least squares, using the equation of a true regression line of (Moore and Edwards, 1965).

\[ y = a + bx \]

where \( a = \bar{y} - bx \)

and \( y = \bar{y} - b(\bar{x} - x) \)

and \( b \), the regression coefficient

\[
\frac{\sum xy - (\sum x)(\sum y)}{n} = \frac{c.s. \ xy}{\sum x^2 - (\sum x)^2/n} = \frac{c.s. \ xy}{c.s. \ xx}
\]

d. Correlation coefficient.

\[
r = \frac{n(\sum xy) - (\sum x)(\sum y)}{\sqrt{(n(\sum x^2) - (\sum x)^2)(n(\sum y^2) - (\sum y)^2)}}
\]

To discover whether a correlation coefficient was significant, the calculated value of \( r \) was compared against tables of \( r \) (Biometrika tables, Table 13) with \( n - 2 \) degrees of freedom.

e. Confidence limits about a regression line, (Moore and Edwards, 1965).

when \( n \) was large (>30) an approximate formula was used:

\[
D = t_{95}^1 \times Sr
\]

where \( Sr \) = residual mean squares
when n was small (≤30) an exact method was used.

The variance about a line when \( x = \bar{x} \) is

\[
\frac{Sr^2}{\chi} = Sr^2 \left( 1 + \frac{1}{n} + \frac{(x - \bar{x})^2}{\overline{xx}} \right)
\]

therefore \( D = t_{0.05}^{1} \frac{Sr^2}{\chi} \)

### 2. Comparison of two regression line slopes (\( b_1 \) and \( b_2 \))

From Moore and Edwards, 1965

First obtain the average about regression line variance -

\[
s^2 = \frac{s_1^2 + s_2^2}{n_1 + n_2 - 4}
\]

where, \( s_1 \) residual sum of the squares for the first regression line

\( s_2 \) residual sum of the squares for the second regression line

Then the variance of the difference between the two slopes is:

\[
s^2_B = \frac{s^2}{\text{c.s. } x_1 x_1} + \frac{s^2}{\text{c.s. } x_2 x_2}
\]

where, \( \text{c.s. } x_1 x_1 \) corrected sums of the squares for first regression line

\( \text{c.s. } x_2 x_2 \) corrected sum of the squares for second regression line

Hence

\[
t = \frac{b_1 - b_2}{s_B}
\]
To find whether $b_1$ is significantly different from $b_2$, this value is compared with tables of $t$ with $v = n_1 + n_2 - 4$ d.f.
Chapter II

DISTRIBUTION OF PROCTOECEAE SUBTENUIS IN THE THAMES ESTUARY AND SURROUNDING AREAS

Freeman and Llewellyn (1958) made no attempt to delimit the occurrence of Proctoeceae along the Thames Estuary to the east or west of Chalkwell, although they did collect infected specimens of S. plana from stations over a distance of about a mile of the mud flats in the neighbourhood of Chalkwell.

Their only other discovery of Proctoeceae from S. plana was made from a sample collected from Whitstable in Kent. Only three Proctoeceae were recovered from one hundred and fifty S. plana examined. They failed to find any Proctoeceae from samples of S. plana collected from the rivers Tamar and Tavy, in Devon (approximately eighty S. plana examined); Dart and Teign, in South Devon (approximately forty examined); the Gwendraeth, in South Wales (ca one hundred examined); Brey, in Suffolk (ca seven); the Essex rivers Colne (seven), and Blackwater (fifty) and from Conway, in North Wales (ca one hundred).

These records of Freeman and Llewellyn (1958) provided the total known distribution of P. subtenuis in Britain, up until 1967, when this work was begun.

The examination, by the present author, of a sample of S. plana
from Chalkwell, in 1967, established that *Proctoeoea* was still present. The extent and the limits of the population, especially in view of the severe winter of 1962/3 which was known to have caused extensive mortalities of the host (Newell, 1964, and Freeman, 1963), were still unknown.

For this reason it was decided that a survey of the *S. plana* collected from different regions of the Thames Estuary and from the neighbouring rivers would be carried out.

**Method of sampling**

At each chosen locality *S. plana* were collected and an estimate of their density recorded. The collecting technique was simply to dig in a suitable area, usually with a fork, and to collect every *S. plana* that was uncovered. This technique was used at every locality and in this way it was hoped that the sample collected was representative (from the point of view of containing *S. plana* of various sizes/ages) of the *S. plana* population in that area.

An estimate of the density of the population of the host was achieved by determining the average number of *S. plana* falling within five 1/4 square metre areas. Because of the fact that the population at any one locality was found to be extremely variable within a small area, the densities quoted are approximate.
Description of the collection sites

The sites from which S. plana were collected are listed below and are also illustrated on the map (Fig. 5). Ordinance survey grid references are given for each site. Some comment is made on the nature of the collecting area and upon the other animals present. This latter point is only concerned with other molluscs and is included because of considerations of the life history of P. subtrunx that will follow (page 71).

River Blackwater, Essex.

1. Tollesbury (972089)

Only isolated specimens of S. plana were found at this site although, judging by the number of empty shells, the population was once far more abundant. The area consisted of a clayey substrate but with some flint and shell gravel deposits. It was on these latter deposits that large number of Mytilus edulis (Linnaeus) were found. Cerastoderma edule (L.), Nassora balthica (L.), Nucella lapillus (L.), Littorina littorea (L.),

Ootrea edulis (L.), Petricola pholadiformis (Lamarck) and the prosobranchs Crepidula fornicata (L.), Littorina littorea (L.),

L. saxatilis (Olivi), and Hydrobia ulvae (Pennant) all occurred fairly commonly.
Other areas in the River Blackwater were visited especially on the southern side but no S. plana were found, although, as at Gislesbury, judging by the number of empty shells, considerable populations presumably did once exist.

River Crouch, Essex

2. Burnham-upon-Crouch (955954)

This was another area of variable substrates: olay, mud and gravel. S. plana was found to be locally common, but with an overall density of about twenty per square metre. Mytilus edulis was present as were C. edule, M. balthica, H. arenaria, P. pholadiformis and C. fomica.

3. North Fambridge (853965)

The substrate here was very soft, fine mud in which the numbers of S. plana were difficult to estimate. They were found to occur in densities of at least twenty per square metre. Very few other animals were evident in this area with the exception of large numbers of the polychaetes Nereis diversicolor (C. F. Müller)

4. South Creekeea (953925)

This location was similar to North Fambridge except that the substrate was coarser. S. plana was locally abundant but overall in
the region of fifty per square metre. Again, the only other animal occurring in large numbers was *N. diversicolor*.

**River Roach, Essex.**

5. **Farraham (913939)**

   The substrate was variable with areas of soft mud and areas of gravel. *S. plana* was common in the areas of mud (ca fifty per square metre). The lamellibranchs *M. edulis*, *M. arenaria*, *C. edule*, *M. balthica*, *P. pholadiformis* and *O. edulis* (artificially laid) and the prosobranch, *G. fimbriata* were all present.

**River Thames, north coast, (Essex)**

6. **Chalkwell (854855)**

   This was found to be an area of mainly mud but with some gravel above mid-tide level. *M. edulis* was abundant, occurring in dense beds at mid-tide level. The lamellibranchs, *M. balthica*, *C. edule* and *M. arenaria* were all common.

   The density of *S. plana* was not found to be as high as recorded by Freeman and Llewellyn (1958) but to be only in the region of twenty per square metre.

7. **Two Tree Island (847826)**

   *S. plana* was uncommon, occurring mainly in the silty mud on the
edge of channels. Very large numbers of empty shells were evident.

*M. balthica* and *M. arenaria* were common.

8. **Leigh Beck (833825)**

*S. plana* was uncommon in the clay substrate found at this locality. The specimens that were collected were generally small.

*M. arenaria, M. edulis, M. balthica* and *C. edule* were present but the most common animal was the polychaete, *M. diversicolor*.

9. **Deadman's Point, Canvey Island (825795)**

The substrate was found to consist of mud overlying gravel.

Very few animals were found with the exception of *M. diversicolor*.

*S. plana* was rare.

10. **River Thames, south coast (Kent)**

11. **Allhallows (842788)**

The substrate was soft mud, sandy in parts. The density of *S. plana*, although locally abundant, was in the region of fifty per square metre overall. Other animals present included *M. balthica*, *C. edule, M. arenaria* and numerous *H. ulvae*.

This was an area of very soft sticky mud. *S. plana* was locally very dense but with an overall density of approximately fifty per square
metre. *M. balthica* and *N. diversicolor* were common.

Other sites on the Kent coast were visited. Attempts to find *S. plana* from the Isle of Grain; in the River Medway; Leysdown, on the Isle of Sheppey; and from Whitstable, in Kent, all proved unsuccessful. The latter was the most disappointing because of the fact that Freeman and Llewellyn (1958) had reported the rare occurrence of *Proctobranchia* from this location. The unsuccessful nature of my own attempts and those of countless students on field courses to find a species that was once abundant (Newell, 1954) may, at least in part, be due to the severe winter of 1962/3 (see Introduction).

Other localities from which *S. plana* were examined

**River Adur, Sussex**

*Shoreham by Sea* (219050)

*S. plana* was found to be fairly common in certain areas, occurring in the region of fifty per square metre. *H. edulis*, *M. balthica* and *S. adune* all occurred, as, in large numbers, did the polychaete *N. diversicolor*.

**River Tamar, Devon**

*Plymouth*

These *S. plana* were supplied by the Marine Biological Station
and therefore exact details as to their density or animal associates were not possible.

Examination

All the *S. plana* collected from the above mentioned sites were transported back to the laboratory, opened and examined with the aid of a binocular microscope to ascertain whether they were infected with *P. subtenuis*.

**TABLE I**

**Distribution of Proctoeces subtenuis in the vicinity of the Thames Estuary**

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number of <em>S. plana</em> examined</th>
<th>Number infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>River Blackwater, Essex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Tollesbury</td>
<td>12</td>
<td>nil</td>
</tr>
<tr>
<td>River Crouch, Essex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. Burnham-on-Crouch</td>
<td>51</td>
<td>nil</td>
</tr>
<tr>
<td>3. North Fambridge</td>
<td>28</td>
<td>nil</td>
</tr>
<tr>
<td>4. South Creeksea</td>
<td>67</td>
<td>nil</td>
</tr>
<tr>
<td>River Roach, Essex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Pagelsham</td>
<td>46</td>
<td>nil</td>
</tr>
<tr>
<td>Locality</td>
<td>Number of S. plana examined</td>
<td>Number infected</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
<td>----------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>River Thames, North Coast, Essex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6. Chalkwell</td>
<td>54</td>
<td>51 (94.4%)</td>
</tr>
<tr>
<td>7. Two Tree Island</td>
<td>33</td>
<td>nil</td>
</tr>
<tr>
<td>8. Leigh Beck</td>
<td>28</td>
<td>nil</td>
</tr>
<tr>
<td>9. Deadman's Point</td>
<td>13</td>
<td>nil</td>
</tr>
<tr>
<td>River Thames, South Coast, Kent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10. Allhallows</td>
<td>97</td>
<td>nil</td>
</tr>
<tr>
<td>11. Harty Ferry</td>
<td>108</td>
<td>nil</td>
</tr>
</tbody>
</table>

Other localities from outside the Thames Estuary from which samples of *S. plana* were examined:

<table>
<thead>
<tr>
<th>Locality</th>
<th>Number of S. plana examined</th>
<th>Number infected</th>
</tr>
</thead>
<tbody>
<tr>
<td>River Adur, Sussex</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Shoreham</td>
<td>50</td>
<td>nil</td>
</tr>
<tr>
<td>River Tamar, Devon</td>
<td>50</td>
<td>nil</td>
</tr>
</tbody>
</table>

Discussion:

As is illustrated by table 1 and Fig. 5, *Proctocephes* was only recovered from *S. plana* that were collected from the vicinity of Chalkwell, on the north coast of the Thames Estuary. This is the same locality from which Freeman and Llewellyn (1958) first described the parasite.
Fig. 5. Distribution of Proctoeces subtenuis in the Thames Estuary and neighbouring areas in 1969.

- S. plana infected by P. subtenuis.
- S. plana uninfected by P. subtenuis.
R. plana collected from all other localities, upon examination, were found to be uninfected; even those from areas that were only relatively a short distance away (for example, Two Tree Island, one mile away).

It was evident from this investigation that the distribution of *P. subtenues* as a parasite of *S. plana* was extremely localised to one area of the Thames Estuary. This was a very similar situation to that recorded by Freeman and Llewellyn (1958), the only difference being that these authors found three Proctoeoes from one hundred and fifty *S. plana* collected from Whitstable, in Kent. As has already been stated, no *S. plana* could be found at Whitstable. Those *S. plana* from Harty Ferry, a short distance from Whitstable, that were examined were all found to be uninfected.

It therefore appeared from this survey that the distribution of Proctoeoes had not extended since 1954. The next step was to determine the extent of the population of the parasite to the east and west of Chalkwell and to attempt to determine the reasons for its lack of spread to neighbouring areas.
Chapter III

ASPECTS OF THE ECOLOGY OF PROCTEDES SUBTEINUS ON THE NORTH COAST OF THE THAMES ESTUARY

I. Distribution

Eight locations along the north coast of the Thames Estuary were selected as areas at which S. plana was known to occur. The names given to these eight locations are the ones used by the author to define a local area and thus the names may not be evident on an ordinance survey map. Map grid references are listed for each site for detailed reference. The eight collecting sites are described in the following section in geographical order from west to east, and are also illustrated on the accompanying map (Fig. 6).

At each of these localities a sample of S. plana was collected in October of each of the three years of the investigation. The method of sampling employed was as previously described (page 35).

The numbers of the sample varied from locality to locality depending upon the density of the host. In 1968 and 1969 a very similar number of S. plana was examined from each locality to ensure that variability of the size of the sample was not, in part, responsible for the observed results.

Each collecting site is briefly described in the following
Fig. 6. Location of collecting sites along the north coast of the Thames Estuary.

(Numbers of collecting sites correspond to those in text)
section and the major animal species found in the same locality
are listed. This list of animals is not intended to constitute a
fauna list for each location but to present an indication of the nature
of the environment by commenting upon the common animals. Possible
intermediate hosts are also indicated by studying the other animals,
especially the molluscs, present. Those species that may migrate
into the area at high tide are excluded because of lack of detailed
knowledge about their numbers and their distribution.

Collecting sites

1. **Benfleet Bridge** (855782)

   This was selected as the most westward site and was a creek
bounded on both sides by salt marshes. The substrate consisted of
very soft mud that afforded a good habitat for the most abundant
animal, *Nereis diversicolor*. The tellinid lamellibranch, *Nacoma*
balthica and the amphipod *Corophium volutator* (Fallan), were also
common.

   A patchy distribution of *S. plana* was found with the overall
density being low: approximately ten per square metre.

2. **Benfleet Creek** (852894)

   This site, approximately one mile from Benfleet Bridge, was found
to be of very variable substrate, ranging from fine silt to "rock".

This variability was due to the accumulation of large amounts of brick rubble in the area dating back to when the jetty at this location was the site of loading of Thames barges with bricks produced in the nearby brick works.

This fact has led to areas of very coarse substrate that present a suitable habitat for the "American Pinidock", *Petricola pholadiformis*, the only occurrence of this lamellibranch that the author has discovered on the north coast of the Thames Estuary, in the vicinity of Southend-on-Sea. The lamellibranch *N. arenaria* was also common amongst the coarse substrate, and *N. diversicolor*, *N. balthica* and *C. volutator* were common where the substrate was finer. The nearby sandy deposits were colonised by *Cerastoderma edule* and the bricks and stones allowed for the attachment of *Mytilus edulis*.

With this very variable substrate the density of *S. plana* was also found to be very variable, being in the region of twenty per square metre.

3. **Leigh Bridge** (853823)

This collecting site was found to be very similar to Benfleet Bridge; the mud was very soft and the crook was bounded by salt marshes. The dominant animals in this area were *N. diversicolor*, *C. volutator*,
M. balthica and Carcinus maenas (L.)

The density of S. plana was high, being in the region of two hundred per square metre for most of the area.

4. Chalkwell Station (654851)

This was another area of soft substrate but, uncommon to the other areas, was covered by a dense growth of the angiosperm Zostera sp. This was chosen as the most westward collecting site actually in the Estuary, as compared to the previously described sites that were situated within creeks and bounded by salt marshes. It was also found to be the beginning of large populations of animals, many of which had not been encountered in any numbers further to the west.

M. balthica occurred in very large numbers as did the prosobranch gastropod Hydrobia ulvae, Littorina littorea, L. saxatilis, L. obtusata (L.) and the lamellibranchs M. arenaria and C. edule were all locally common. Isolated, small clumps of M. edulis also occurred.

The density of S. plana, although very variable over the wide expanses of the intertidal zone, was found to attain densities in excess of fifty per square metre.

5. Chalkwell Bowling Green (655854)

The substrate was discovered to be somewhat coarser than further to
to the west, *H. edulis* was very common inshore as isolated clumps and at mid-tide level were the beginnings of the large mussel beds that extended eastwards. *H. balthica, H. arenaria* and *C. edule* were all common.

The density of *S. plana* was found to be in the region of twenty per square metre. This is compared to the estimate of Freeman and Llewellyn (1958) of a density of between five hundred and one thousand per square metre. Clearly the population had not recovered from the effects of the severe winter of 1962/3 (see Page 23).

6. Crowstone (852859)

It was in this area that the inter-tidal zone was found to attain its greatest width and here also *S. plana* was found to have its widest distribution, occurring in the muds well below mid-tide level and up into the gravel and sandy areas above mid-tide level. The greatest densities of *S. plana* (ca. two hundred per square metre) occurred just below mid-tide level, in and amongst the large beds of *Mytilus*. The fauna was found to be rich in this area with many polychaetes and crustaceans present. The lamellibranchs *H. balthica, C. edule, H. arenaria* as well as *H. edulis* were all very common. The prosobranchs *L. littorea, L. saxatilis, L. obtusata, H. ulvae* and *Crepidula fornicatea* were all locally common.
7. Clifftown (650874)

This was another area where the density of *S. plana* was found
to be high, being in parts, in the region of two hundred per square metre.
The area was very similar, both in substrate and in the animal species
found, to area 6 (Crowstone), with the limellibranchs *M. edulis*,
*M. balthica* and *M. arenaria* being particularly common.

8. Thorpe Bay (657907)

This was the most eastward site from which *S. plana* were found
although others were investigated. The substrate was a mixture of both
sand and mud, and for this reason a variety of animals were found to
occur. *C. edule* was abundant in the sandy areas, whereas *M. balthica*
and *M. arenaria* tended to be found in the more muddy area alongside the
*S. plana*. *M. edulis* occurred in isolated clumps and the prosobranchs,
*L. littorea*, *L. saxatilis* and *H. ulvae* were not uncommon. The overall
density of *S. plana* was low, being in the region of twenty per square metre.

Results:

The samples of *S. plana*, once collected, were transported back to the
laboratory and opened. With the aid of a binocular microscope the number
of parasites in each kidney was ascertained.

The results from such surveys carried out in 1967, 1968 and 1969 are
expressed in Table 2 and Fig 7.
<table>
<thead>
<tr>
<th>Site Number</th>
<th>Location</th>
<th>No. of Planas examined infected S. planae</th>
<th>% of S. planae examined infected S. planae</th>
<th>Standard error of mean</th>
<th>Standard error of mean</th>
<th>Standard error of mean</th>
<th>Standard error of mean</th>
<th>Standard error of mean</th>
<th>Standard error of mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Benfleet Bridge</td>
<td>20</td>
<td>5%</td>
<td>0.05</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
</tr>
<tr>
<td>2</td>
<td>Benfleet Creek</td>
<td>20</td>
<td>20%</td>
<td>0.12</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
</tr>
<tr>
<td>3</td>
<td>Leigh Bridge</td>
<td>28</td>
<td>7.1%</td>
<td>0.05</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
<td>0.026</td>
</tr>
<tr>
<td>4</td>
<td>Chalkwell Station</td>
<td>40</td>
<td>42.5%</td>
<td>0.57</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>5</td>
<td>Chalkwell Bowling Green</td>
<td>10</td>
<td>70.5%</td>
<td>43</td>
<td>0.42</td>
<td>0.42</td>
<td>0.42</td>
<td>0.42</td>
<td>0.42</td>
</tr>
<tr>
<td>6</td>
<td>Crowstone</td>
<td>32</td>
<td>65.6%</td>
<td>43</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
<td>0.20</td>
</tr>
<tr>
<td>7</td>
<td>Cliffe Point</td>
<td>26</td>
<td>92.3%</td>
<td>55</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
<td>0.28</td>
</tr>
<tr>
<td>8</td>
<td>Thorpe Bay</td>
<td>12</td>
<td>16.6%</td>
<td>37</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
<td>0.11</td>
</tr>
</tbody>
</table>
Fig. 7. Distribution of Proctoececs subtenuis along the north coast of the Thames Estuary.
Discussion:

The main point that is evident from Table 2 and (Fig 7) is the extreme localisation of infection, and the fact that this pattern was repeated, with only minor variation, in all three years of study. Thus the _S. plana_ collected from sites 4, 5, 6, and 7 (from Chalkwell Station to Clifftown) were always more heavily infected than those collected from the other sites. The peak of infection appeared to be at Clifftown (site 7) and as samples were examined that had been collected from sites to the east or west of Clifftown, there was discovered to be a reduction in the level of infection. An anomaly appears to be the fact that the _S. plana_ collected from Benfleet Creek (site 2) were consistently and significantly (P<0.05) more heavily infected than the _S. plana_ collected from the sites of either side which were only rarely infected.

This pattern of distribution had little relation to the density of _S. plana_ at each locality. For example, the density of _S. plana_ at Leigh Bridge was very similar to that at Clifftown (ca two hundred per square metre) but the _S. plana_ from Leigh Bridge were only rarely infected whereas those from Clifftown were always heavily infected.

It could be suggested that the observed pattern of distribution was brought about by some physical factor, or factors, acting upon the
larval or adult trematode. This appears unlikely because it is difficult to conceive that such factors would vary sufficiently within short distances. Certainly, salinity, which was probably the obvious one to examine was very similar at sites 4, 5, 6, 7 and 8 which were all in the Estuary proper. It has also been demonstrated, as will be discussed in a later section (page 156), that the adult trematodes are unaffected by salinity changes larger than occur in the area.

It appears most likely that this observed pattern of distribution of the adult trematode could be due to the fact that there were more larval stages of Prootooeces at sites 4, 5, 6 and 7 than at the others. This could have been due to tidal currents carrying the larval stages to certain areas more than others or to the fact that the first intermediate host was more abundant at sites 4, 5, 6 and 7 and therefore there were more stages at these areas. If there needs to be a close association between the two hosts, possibly due to a lack of dispersal power of the larval Prootooeces, then it would mean that only at those sites where there was a population of the first intermediate host would there be the likelihood of a high level of infection of the S. plana.

The suggestion that the first intermediate host occurred at Benfleet Creek (site 2) but not at Benfleet Bridge or Leigh Bridge, the sites on
either side, could also explain the higher level of infection discovered at this locality.

This hypothesis has been mentioned here as a possible explanation of the observed distribution of Proctoeees and will be discussed in more detail in a subsequent section dealing with the life history.

As well as this pattern of distribution that is so evident, the results clearly show that Proctoeees was very successful as a parasite of S. plana on the north coast of the Thames Estuary. This was especially so at sites 5, 6 and 7 where in 1969 over 90% of all S. plana collected were infected and with an average of between three and four Proctoeees per host. The maximum number of Proctoeees found in a single S. plana was fourteen and this was found on two occasions, both S. plana having been collected from Clifftown.

The high numbers of Proctoeees found from S. plana collected from sites 5, 6 and 7 demonstrate that the parasite was very successful in this area. The fact that all sizes of Proctoeees from small transparent ones to large, red, gravid ones were found, and that the level of infection of the S. plana collected from sites 4, 5, 6 and 7 had increased significantly during the three years of study, was a good indication that the life cycle was being successfully maintained in the
area. Thus larval Proctoeces were being continually released from some undetermined intermediate host.

2. Increase in the level of infection of S. plana by Proctoeces

The fact that the level of infection of the S. plana collected from sites 4, 5, 6 and 7 had increased during the period of study can be further illustrated by reference to table 3 and fig. 8. These are the results obtained from samples, collected at intervals, from Clifftown (site 7).

Discussion

It is evident that from a level of infection of the S. plana of between two and three Proctoeces per host in 1967 an increase occurred to an infection level of between four to six per host in 1969 and 1970 (P < 0.05. This was for a comparison of the results of October 1967 and October 1969 – see Statistical Test b, Page 30). This latter level of infection is similar to that recorded by Freeman and Llewellyn (1958) for S. plana collected from Chalkwell (site 5) and so it may be that the infection had increased to a level similar to that which occurred prior to the 1962/3 winter.

The variation in the level of infection of the samples of S. plana that were collected after August 1969, and in particular the low results
<table>
<thead>
<tr>
<th>Month and year of sample</th>
<th>Number of S. plane examined</th>
<th>% of S. plane infected</th>
<th>Average number of Proctoeospora per S. plane</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>October 1967</td>
<td>21</td>
<td>100</td>
<td>2.7</td>
<td>0.35</td>
</tr>
<tr>
<td>November</td>
<td>26</td>
<td>92.3</td>
<td>2.15</td>
<td>0.28</td>
</tr>
<tr>
<td>December</td>
<td>37</td>
<td>94.6</td>
<td>2.6</td>
<td>0.27</td>
</tr>
<tr>
<td>January 1968</td>
<td>48</td>
<td>95.8</td>
<td>2.75</td>
<td>0.26</td>
</tr>
<tr>
<td>February</td>
<td>139</td>
<td>94.5</td>
<td>2.6</td>
<td>0.20</td>
</tr>
<tr>
<td>March</td>
<td>110</td>
<td>94.6</td>
<td>2.6</td>
<td>0.17</td>
</tr>
<tr>
<td>April</td>
<td>58</td>
<td>87</td>
<td>2.3</td>
<td>0.19</td>
</tr>
<tr>
<td>May</td>
<td>72</td>
<td>91.5</td>
<td>2.7</td>
<td>0.21</td>
</tr>
<tr>
<td>August</td>
<td>61</td>
<td>98.3</td>
<td>3.1</td>
<td>0.22</td>
</tr>
<tr>
<td>October</td>
<td>50</td>
<td>98</td>
<td>3.6</td>
<td>0.28</td>
</tr>
<tr>
<td>November</td>
<td>55</td>
<td>100</td>
<td>3.9</td>
<td>0.29</td>
</tr>
<tr>
<td>December</td>
<td>30</td>
<td>100</td>
<td>4.5</td>
<td>0.44</td>
</tr>
<tr>
<td>January 1969</td>
<td>40</td>
<td>100</td>
<td>4.15</td>
<td>0.33</td>
</tr>
<tr>
<td>February</td>
<td>30</td>
<td>96.6</td>
<td>5.3</td>
<td>0.42</td>
</tr>
<tr>
<td>March</td>
<td>40</td>
<td>100</td>
<td>4.35</td>
<td>0.27</td>
</tr>
<tr>
<td>April</td>
<td>50</td>
<td>100</td>
<td>5.1</td>
<td>0.41</td>
</tr>
<tr>
<td>May</td>
<td>40</td>
<td>100</td>
<td>5.7</td>
<td>0.27</td>
</tr>
<tr>
<td>June</td>
<td>6</td>
<td>100</td>
<td>5.6</td>
<td>0.68</td>
</tr>
<tr>
<td>July</td>
<td>40</td>
<td>100</td>
<td>6.1</td>
<td>0.46</td>
</tr>
<tr>
<td>August</td>
<td>20</td>
<td>95</td>
<td>5.95</td>
<td>0.75</td>
</tr>
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<td>26</td>
<td>96.1</td>
<td>5.0</td>
<td>0.57</td>
</tr>
<tr>
<td>October</td>
<td>47</td>
<td>98</td>
<td>4.4</td>
<td>0.42</td>
</tr>
<tr>
<td>November</td>
<td>18</td>
<td>100</td>
<td>6.4</td>
<td>0.49</td>
</tr>
<tr>
<td>Month and year of sample</td>
<td>Number of \textit{S. plana} examined</td>
<td>% of \textit{S. plana} infected</td>
<td>Average number of Proctoces per \textit{S. plana}</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>-------------------------</td>
<td>-------------------------------------</td>
<td>---------------------------------</td>
<td>-------------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>January 1970</td>
<td>21</td>
<td>95</td>
<td>5.7</td>
<td>0.73</td>
</tr>
<tr>
<td>May</td>
<td>50</td>
<td>84</td>
<td>4.1</td>
<td>0.49</td>
</tr>
</tbody>
</table>
Fig. 8. Change in level of infection of S. plana from Clifftown.
for October 1969 (P = 5) and May 1970 (P = 5), as compared with the result for August 1969 (see Statistical Technique b, Page 30), are difficult to explain, although it is considered that it was most probably associated with a change in the population of the host.

Since about August 1969, the Clifftown population of S. plana has, as judged by the increasing difficulty with which samples of S. plana were collected, declined in numbers. The reason for this is uncertain although the fairly severe winter of 1969/70 could have possibly accounted for some of the mortalities observed at the end of 1969 and beginning of 1970.

The decline of the population of the S. plana could have brought about a variation of the population of the parasite by a combination of ways: Adverse conditions, such as severe low temperatures, may have preferentially killed the more heavily infected S. plana because of their greater parasite burden. The fact that this was unlikely was illustrated by the occurrence in the May 1970 sample (50 S. plana) of one S. plana that contained fourteen Proctocetes, one that contained thirteen, one eleven, one ten and four with nine.

It is possible that the population of S. plana at Clifftown may have been going through a period of natural senescence with many of
the older *S. plana* dying and being replaced by younger *S. plana*. As these younger/smaller *S. plana* were less heavily infected (see page 66) then an increase in their number in the sample would have greatly reduced the overall number of *Protoeoees* per host for that sample. This was seen to be at least partially true for the May 1970 sample, where 10% of the sample consisted of *S. plana* under 1.8 cm in length.

Similarly the October 1969 sample, another sample with an overall lower level of infection as compared with the ones before and after, had 8% of the *S. plana* under 1.8 cm. This was in comparison with other samples, for example November 1968, which only contained 4% of the *S. plana* under 1.8 cm.

As these small *S. plana*, under 1.8 cm, only usually contained one *Protoeoees* (see page 67) then an increase in their number in a sample would have brought about an overall decrease in the number of *Protoeoees* recovered.

A combination of these factors could have partially accounted for the observed low results of October 1969 and May 1970. The heterogeneous nature of the host population with many small, low infected *S. plana* and many larger, heavily infected *S. plana* could have brought about variations in the infection level of a sample.
depending on the ratio of one to the other collected.

Even taking these variations into account it is very evident that the overall level of infection of the population of \textit{S. plana} at Clifftown had increased over the period of the study. This is important because it further illustrates that the parasite was extremely successful in this area and that the life cycle was well established, resulting in a continued chance of re-infection of the \textit{S. plana} by larval \textit{Protoeea} released from some intermediate host.

3. Relation between the size of the host and number of \textit{Protoeea}

\textbf{Method}

When the samples of \textit{S. plana} collected from Clifftown were examined, the length of each \textit{S. plana} was measured to the nearest 0.5 mm (1.7\% of an average \textit{S. plana} of 3.00 cm length) and the number of \textit{Protoeea} occurring in each was recorded. The information thus gathered is illustrated in table 4 and fig. 9. The grouping of the \textit{S. plana} so examined into arbitrary size groups allowed for the plotting of histograms (table 5 and fig. 10) which demonstrate the percentage of a certain number of \textit{Protoeea} that were found in each size group.

\textbf{Discussion}

From table 4 and fig. 9, it is evident that the level of
### Table 4: Relation between Size of Host and Number of Proctoeces

<table>
<thead>
<tr>
<th>Length of <em>S. plane</em> (cm)</th>
<th>Number of that size examined</th>
<th>Total number of Proctoeces recovered</th>
<th>Average number of Proctoeces per that size host</th>
<th>Standard error of the mean</th>
</tr>
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</tr>
<tr>
<td>0.85</td>
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<td>-</td>
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<tr>
<td>0.95</td>
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<td>1.10</td>
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<td>1.15</td>
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<td>-</td>
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<td>0</td>
<td>-</td>
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<td>0</td>
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</tr>
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<td>-</td>
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<td>-</td>
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</tr>
<tr>
<td>Length of S. plana (cm)</td>
<td>Number of that size examined</td>
<td>Total number of Protozoa recovered</td>
<td>Average number of Protozoa per that size host</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>------------------------</td>
<td>------------------------------</td>
<td>------------------------------------</td>
<td>---------------------------------------------</td>
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</tr>
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<td>1</td>
<td>1</td>
<td>±0.40</td>
</tr>
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<td>50</td>
<td>2.6</td>
<td>±0.63</td>
</tr>
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<td>±0.30</td>
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<td>±0.45</td>
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</tr>
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<td>-</td>
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<td>13</td>
<td>6.5</td>
<td>-</td>
</tr>
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</table>
Fig. 2. Relation between size of host and number of protozoa.

AV. number of protozoa per g. plane.
<table>
<thead>
<tr>
<th>Size Range of S. Plan (Length in cm)</th>
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<th>1</th>
<th>2</th>
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<th>6</th>
<th>7</th>
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<th>11</th>
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<td>0.6 - 1.20</td>
<td>62.5</td>
<td>37.5</td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1.25 - 1.80</td>
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<td>50</td>
<td>10</td>
<td></td>
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<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>1.85 - 2.40</td>
<td>16.7</td>
<td>25.1</td>
<td>33.5</td>
<td>8.3</td>
<td>4.1</td>
<td>2.0</td>
<td>6.2</td>
<td>4.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
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<td>2.45 - 3.00</td>
<td>4.5</td>
<td>13.2</td>
<td>20.5</td>
<td>19.8</td>
<td>17.0</td>
<td>9.5</td>
<td>5.4</td>
<td>6.5</td>
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<td></td>
</tr>
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<td>3.05 - 3.60</td>
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<td>8.4</td>
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<td>1.8</td>
<td>1.5</td>
<td>0.6</td>
<td>1.2</td>
<td>0.6</td>
</tr>
</tbody>
</table>
infection increased with the size/age of the host. Thus as the S. plana was exposed to larval stages there was a continual likelihood of re-infection. It is interesting that this data also provide evidence that S. plana can become infected by Proctoeces at an early stage in its life. A single, small, immature Proctoeces was found in a S. plana of 0.85 cms length; that is about one year old.

The histograms (fig. 10) demonstrate that as the S. plana increased in size/age, the more Proctoeces they contained and the greater were the number of hosts that contained a large number of Proctoeces. Thus the 0.6 - 1.2 cms. length group of S. plana never contained more than a single Proctoeces and they usually contained none. With the increase in the host size group it can be seen that the numbers of Proctoeces that most commonly occurred in a single host also increased. Thus the largest S. plana, the 3.05 - 3.60 cms. group, were rarely uninfected and most of the S. plana contained four Proctoeces. A proportion of the S. plana in this size group also contained large numbers of Proctoeces; up to fourteen in a single host.

From both of these sets of data it is evident that infection can continue as the S. plana grows older. On this basis infection by one Proctoeces does not appear to encourage or discourage the infection by
another Proctoeces. Also any suggestion that the growth of the host
and growth of the renal pore may facilitate loss of Proctoeces by the
host appears to be unfounded, at least in the size ranges found.

Summary of the results of the distribution of Proctoeces

From this work on the distribution of Proctoeces in the region of
the Thames Estuary, it is clearly evident that the endemic areas of
Proctoeces were extremely localised. It is also clear that Proctoeces
as a parasite of S. plana was extremely successful in certain regions:

between Chalkwell Station and Clifftown, where nearly every S. plana
collected was found to be infected, with an average of between three
and four Proctoeces.

This and the fact that the level of infection had increased over
the period of study and that infection of S. plana appeared to continue
with increase in the size/age of the host emphasises that the life cycle
of Proctoeces was being successfully maintained. The high level of
infection and its increase must have been due to the continued release
of larval stages of this trematode from some intermediate host.
Chapter IV

THE LIFE HISTORY OF PROCTOECES SUBTENUIS IN THE
THAMES ESTUARY

The life history of Proctoeces, when the adult is in the hind gut of a fish, has been suggested by Yamaguti (1958) to involve a molluscan intermediate host that produces cercariae and then a second intermediate host in which unencysted metacercariae occur, transference of this metacercaria to the fish occurring when the latter eats the mollusc.

This is somewhat borne out by the fact that the fish so far recorded as harbouring Proctoeces are bottom feeders, armed with strong jaw and pharyngeal teeth which are used for crushing molluscs. The majority of the fish that have been recorded as being hosts for Proctoeces belong to the families Sparidae and Labridae.

There is no evidence so far available that a fish host is involved in the life history of Proctoeces in the Thames Estuary, although members of these two families may occur there. Wheeler (1969) in his book "Fishes of the British Isles and north western Europe", indicates that certain sparids: the Bogus, Boops boops (Linnaeus, 1758); the Red Sea Bream, Pagellus bogaraveo (Brünich, 1768); and the Black Sea Bream, Spondyliaus australis (Linnaeus, 1758) occasionally migrate into the North Sea from the English Channel. The diet of these fish is not
well known although it is likely that they do feed on molluscs to some extent.

The diet of the Labridae is better known and is listed by Wheeler (1969) as including a variety of invertebrates including mussels, winkles and whelks. Although Wrasse are more common on rocky shores than on sandy or muddy ones, the Ballan Wrasse, Labrus bergylta (Ascanius 1767); and the Corkwing, Crenilabrus melops (Linnæus, 1758) are fairly common in the general area of the Thames Estuary.

Freeman (1962a) demonstrated that the Wrasse, Crenilabrus melops and Otolabrus rupestris (Linnæus, 1758) will act as host for P. subtenus for short periods, although no difference was detectable between parasites obtained directly from S. plana and those that had been in the fish host. It thus does not appear to be necessary for the parasite to enter a fish in order to reach a state of maturity. This is further suggested by the fact that the Prootoeces in the mollusc produce fertile eggs with motile ciliated miracidia.

Even though the food of Wrasse, that could possibly occur in the area, may include molluscs, it is unlikely that S. plana living as it does well below the surface of the mud would be readily available for food for fish.
Further and almost conclusive evidence of the lack of a fish host in the life history of Prootoeeoes in the Thames Estuary is provided by the localisation of the parasite to the north coast. Spread to neighbouring areas where S. plana is abundant would be facilitated if a motile fish host was involved. It therefore appears very unlikely that a fish host is involved in the life history of Prootoeeoes in the Thames Estuary, although the existence of members of the Labridae and Sparidae in the area must be taken into account in the final analysis, especially if the first intermediate host is more readily available as food for these fish.

It is clear that the heavy infection of the S. plana by Prootoeeoes at the sites in the vicinity of Southend must be maintained by larval stages developing in an invertebrate host. The life history of one member of the family Fellodistomatidae, Bacciger bacciger has been known since it was first described by Palombi (1934). In this case and in the case of the cercaria described by Martin (1945) and redescribed by Cable (1954), which undoubtedly belongs to the same family, the cercaria was non-ocellate and trichocercous, developing in sporocysts in marine lamellibranchs. Bacciger bacciger belongs to the sub-family Fellodistomatinae whereas Prootoeeoes belongs to the sub-family
Haplocladinae. Cable (1954) suggested that the larvae in this latter
sub-family may be furcocercous or trichofurcocercous.

Uezmann (1953) described a microcercous cercaria developing within
orange-pigmented sporocysts that occurred in the gonad and mantle tissue
of Mytilus edulis from certain areas of America. He tentatively
referred this larval digenean to the family Fellodistomatidae on the
basis of finding progenetic larvae of the genus Prooctoces in the
visceral mass of three of the infected mussels.

The tail-less cercariae described by Hopkins (1954) as developing
within orange pigmented sporocysts which occupied part of the mantle of
the Hooked mussel, Brachiodontis recurvus from Louisiana, were also
assigned, by her, to the family Fellodistomatidae. Stunkard and Uezmann
(1959) considered Cercaria brachiodontis, as described by Hopkins (1954),
to be so morphologically similar to Cercaria milfordensis of Uezmann (1953)
that "their relationship was immediately recognisable".

These discoveries by Uezmann (1953) and Hopkins (1954) of
microcercous or tail-less larvae of members of the family Fellodistomatidae
demonstrated the remarks on caudal structure expressed by Cable (1954).

He could see "no difficulty in the interpretation of the caudal structure
of many fellostomatid cercariae in which the tail is reduced or even
absent". Cable considered that such instances of caudal reduction were associated with the abbreviation of free-living activity now recognised to occur in various distantly related families.

The discovery of microcercous larvae by Uzmann (1953) and Hopkins (1954) and more particularly the description of the complete life history of Proctoeces from Mytilus edulis by Stunkard and Uzmann (1959) added greatly to the knowledge of the sub-family Haplocladinace.

Stunkard and Uzmann (1959) demonstrated that the microcercous larvae developed within simple sacculate, motile, orange-pigmented sporocysts that were found primarily within the blood sinuses and interfollicular lymph spaces of the mantle gonad, where infection led to a suppression of gametogenesis or complete atrophy of the reproductive organs.

The cercarial bearing sporocyst generations were preceded by and also accompanied by smaller, non-pigmented, sporocysts which were observed to contain undifferentiated germ balls. The discovery of unencysted metacercariae and of developmental stages from cercariae to gravid adults was taken to demonstrate that Cercaria milfordensis as described by Uzmann (1953) was the larval stage of a species of Proctoeces, the complete life history of which occurred within
**Mytilus edulis**

A similar situation of the complete life history occurring within the one molluscan host has been suggested by Loos-Franck (1969) for a species she calls *P. bucoini* from the digestive gland of the whelk *Buccinum undatum*. This statement is made without any evidence of intermediate stages but on the basis that a large number of Prootoeoes occur per whelk (up to one hundred and eighty) although there is an overall low level of infection of the population (five out of ninety three whelks). She reasons that if infection was to come from cercarial invasion then a higher percentage of the host would be parasitized and there would be fewer Prootoeoes per individual.

A point that she seems not to have considered is that the common whelk, *Buccinum undatum*, is a carnivore with a tendency to scavenging. *Buccinum* is known to attack a variety of bivalves, including *Cerastoderma, Pecten, Mytilus, Ostrea*, and *Mya* (Fretter and Graham, 1962). If one of the bivalves attacked by *Buccinum* was infected with the sporocysts and cercariae of *Prootoeoes* then its ingestion could possibly lead to a heavy infection of the whelk by this parasite. This could possibly explain how a few members of the *Buccinum* population could become heavily parasitised with sexually mature worms.
It is certain that the complete life cycle of Protopoeces does not occur in S. plana. The investigation of almost a thousand S. plana by Freeman and Llewellyn (1958) and subsequently of over two thousand by the present author has failed to yield any cercarial or sporocyst stages of Protopoeces. The furcocercous cercariae developing within sporocysts in S. plana reported by Freeman and Llewellyn (1958) and by Loose-Frank (1969) were most probably the sporocysts of Cercaria dichotoma.

Sporocysts of this gymnophallid (fig. 11) have been found by the present author in S. plana collected from South Crocksea (1 S. plana infected out of 67), from Leigh Beck (1 S. plana infected out of 27), and from Harty Ferry in Kent (4 S. plana infected out of 108) but not from any of the S. plana collected from the sites where Protopoeces occurs. As was suggested by Freeman and Llewellyn (1958), this furcocercous cercaria is in no way connected with the life history of Protopoeces in the Thames Estuary.

From the foregoing discussion it can be concluded that another invertebrate is involved in the life history of Protopoeces in the Thames Estuary. Up to the present, stages of Protopoeces, either described as progenetic metacercariae or as adults, have been described
Fig. 11. *Cercaria dichotoma* from *Scrobicularia plana*.
from lamellibranch and prosobranch molluscs from many parts of the world.

<table>
<thead>
<tr>
<th>Host</th>
<th>Locality</th>
<th>Author</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lamellibranchiata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ostrea gigas</td>
<td>Japan</td>
<td>Fujita, 1925.</td>
</tr>
<tr>
<td></td>
<td>Germany</td>
<td>Loos-Frank, 1969.</td>
</tr>
<tr>
<td>Prosobranchiata</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Turbo cornutus</td>
<td>Japan</td>
<td>Ichihara, 1965.</td>
</tr>
<tr>
<td>Hissoc splendidus</td>
<td>Russia</td>
<td>Dolgyok, 1965 and 1967.</td>
</tr>
<tr>
<td>Buccinum undatum</td>
<td>Germany</td>
<td>Loos-Frank, 1969.</td>
</tr>
<tr>
<td></td>
<td>Scotland</td>
<td>Wooton (pers. comm.)</td>
</tr>
</tbody>
</table>

Larval stages of Prootocoen have been recorded from the mytilid lamellibranch, Prechiodontia rennaueri from Japan, (Yamaguti, 1938) and Mytilus edulis from America (Uzmann, 1953; Stunkard and Uzmann, 1959).

The larval stage of an unknown member of the sub-family Naplocladinae, possibly Prootocoen, has been described from another mytilid,
Eo.

Brachiodontia recurvus (Hopkins, 1954).

As with most other digeneans the first intermediate host of Proctoeces in the Thames Estuary is most probably a mollusc, either a member of the Prosobranchiata or Lamellibranchiata.

1. Distribution of other molluscs in the Thames Estuary

The localisation of the distribution of Proctoeces that was evident from the survey of Sorobicularia from the eight sites on the north coast of the Thames Estuary (see page 53) could be explained if the larval stages of Proctoeces had limited powers of dispersal. In this case there would need to be a close association between the first intermediate host and the S. plana for there to be a good chance of infection of the latter. Thus in areas on the north coast of the Thames Estuary where the first intermediate host was abundant the S. plana would be likely to become infected. S. plana at sites where the intermediate host was rare or absent would be correspondingly less infected.

The finding by Uzmann (1953) and by Stunkard and Uzmann (1959) that the larval stage of Proctoeces was a microcercous cercaria enhances this hypothesis. As Cable (1954) has stated, this type of tail-less cercaria is usually associated with an abbreviation of free-living
activity. Usmann (1953) pointed out that movement of the micro-
cercous cercaria of *Proctoeces* appeared to be restricted to periodic
contraction and elongation which effected a serpentine but random
progress across the substrate. Thus the dispersal of these micro-
cercous cercariae would undoubtedly be impaired and make a close
association of the two hosts obligatory for a successful continuance
of the life history.

Although other factors may also be involved in causing the
pattern of distribution of *Proctoeces* that has been demonstrated,
it is considered that there is reasonable evidence to support the
contention that the distribution of the first intermediate host could
be mainly responsible for the observed pattern.

It was for this reason that the other molluscs occurring at
each collecting site were listed previously (pp.47-). More detailed
discussion will now be devoted to certain species.

*Introduced species*

If members of the genus *Proctoeces* are to be regarded as
normally parasites of the hind gut of fish from Asian, Australasian,
Eastern American, Mediterranean waters and the Black Sea, it could
be argued that *Proctoeces* has been introduced into the Thames Estuary
from overseas. The limitation of the species to the region of the 
Thames Estuary, in a lamellibranch widely distributed in estuarine 
muds around the British Isles, could be taken as an indication of a 
recent introduction. The accidental introduction or migration of the 
normal fish host into the area is a less likely explanation than the 
suggestion that the mollusc that is its first intermediate host may have 
been accidentally introduced.

If an infected intermediate host was introduced, the larval stages 
of Protoocoec, once released, may have been 'sucked up' by Scrobicularia 
and for some reason been able to survive in the kidney of this mollusc, 
the life cycle continuing if the introduced species established itself 
in the area.

Introduced species do exist in the region of the Thames Estuary 
and so these are considered first.

*Patriciola pholadiformis*

This species was recently introduced from the east coast of North 
America (Newell, 1954) and since becoming established in about 1890 it 
now has a limited distribution in the southern and eastern parts of 
Britain (Duval, 1963). Freeman and Llewellyn (1958) reported an 
observation of Duval of a trichocercous cercaria in *Patriciola.*
The present investigation makes it unlikely that *P. pholadiformis* is involved in the life cycle of *Protozoa* in this region. The only location on the north coast of the Thames Estuary from which *Petricola* has been discovered by the present author is Benfleet Creek (site 2) and here there is only a very limited population. The level of infection of the *B. plana* with *Protozoa* at site 2, although higher than at sites 1 and 3 (*P* < 0.05) is still low in comparison with other sites where *Petricola* is absent (5, 6 and 7) (*P* < 0.05). This, coupled with the complete absence of *Protozoa* (fig. 5) from areas where *Petricola* is common: Tollesbury, River Blackwater; Paglesham, River Roach; Creeksea and other localities in the River Crouch (Duval, 1963) makes it unlikely that *P. pholadiformis* is involved in the life history of *Protozoa* in the Thames Estuary.

*Crepidula fornicata*

Although occurring in the Southend area, this prosobranch, which was introduced in the company of oysters, is far more abundant in the Rivers Crouch, Roach and Blackwater where it constitutes a pest to Oyster cultivation. The same applies to the Oyster sting, *Urosalpinx cinerea* which is also far more abundant in these areas in which *Protozoa* has not been found.
Although this is by no means conclusive evidence it is a strong suggestion that these species are not involved in the life history. In the case of *Crepidula*, the only one of the three to occur in reasonable numbers in the Southend area, the investigation of twenty four of this species, collected in the region of infected *S. plana*, for larval trematodes proved negative.

**Indigenous molluscan species**

The majority of the indigenous molluscan species were found to be abundant at localities where the parasite was absent or conversely to be absent or rare where the parasite was most common. For this reason it seemed unlikely that the prosobranchs, *Littorina littorea*, *L. saxatilis*, *L. obtusata* or *Hydrobia ulvae* were involved in the life cycle of *Protozoos* in the Thames Estuary.

The same applied to the lamellibranchs, *Cerastoderma edule*, *Mya arenaria* and *Macoma balthica*.

This qualitative survey obviously only afforded circumstantial evidence that these species were not involved in the life cycle but as has been previously stated, the extreme localisation of the infection of the *S. plana* by *Protozoos* would suggest that a close association would most probably have to exist between *S. plana* and the first intermediate.
host. None of the aforementioned molluscs appeared to have this similarity of distribution.

The results of this qualitative survey of the distribution of other molluscs at each locality suggested that only one mollusc had a similar pattern of distribution to that of Prooctoaes. This mollusc was the Lamellibranch, Mytilus edulis.

**Distribution of Mytilus edulis**

As Freeman (1962a) had pointed out, the finding of microcercous larvae of Prooctoaes in Mytilus edulis in America, shifted the emphasis of the search for infective stages to Mytilids in Britain.

Initial investigations into the likelihood of M. edulis being the intermediate host in the Thames Estuary were carried out early on in the research programme, but none of three hundred M. edulis, collected from an area where Prooctoaes was common, was found to contain larval trematodes that could be considered to be related to Prooctoaes.

At a later date in the research programme, the qualitative appraisal of the distribution of Mytilus at each collecting site was followed by a survey involving the use of 1/4 square metre quadrats. The mean number of Mytilus edulis per square metre was ascertained by averaging the number of Mytilus edulis falling within the confines of
ten quadrats thrown in the vicinity of each collecting site, and multiplying the answer by 4.

Results

<table>
<thead>
<tr>
<th>Site Number</th>
<th>Location</th>
<th>Average number of M. edulis per sq. metre</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Bonfleet Bridge</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>Benfleet Creek</td>
<td>63</td>
</tr>
<tr>
<td>3</td>
<td>Leigh Bridge</td>
<td>0</td>
</tr>
<tr>
<td>4</td>
<td>Chalkwell Station</td>
<td>10</td>
</tr>
<tr>
<td>5</td>
<td>Chalkwell Bowling Green</td>
<td>400</td>
</tr>
<tr>
<td>6</td>
<td>Crowstone</td>
<td>600</td>
</tr>
<tr>
<td>7</td>
<td>Clifftown</td>
<td>540</td>
</tr>
<tr>
<td>8</td>
<td>Thorpe Bay</td>
<td>10</td>
</tr>
</tbody>
</table>

Discussion

From table 6 and Fig. 12 it can be seen that the pattern of the distribution of *Mytilus edulis* closely resembles the pattern of distribution of *Proctoceros*. Most noticeable is the complete absence
Fig. 12. Density of *Mytilus edulis* at collecting sites.
of *Mytilus* from Benfleet Bridge and Leigh Bridge where *Prootoceces*
was only very rarely found. The higher level of infection of *S. plana*
by *Prootoceces* (20% - 33%) at Benfleet Creek corresponded to an
occurrence of *M. edulis*. Thus not only was there an increase in the
population of the parasite at Benfleet Creek but there was also a
population of *Mytilus edulis*.

At Chalkwell Station (site 4), isolated *M. edulis* were found to be
scattered over a wide area of the intertidal zone, but it was at
Chalkwell Bowling Green (site 5), that the main mussel banks began.
These mussel banks extended to the east, to beyond Clifftown (site 7).
The density of these mussel banks was very variable but it was
usually in the region of 500 per square metre. They became less dense
to the east of Clifftown and at Thorpe Bay (site 8) only scattered
mussel clumps remained.

The distribution of *Mytilus edulis* and the distribution of
*Prootoceces* clearly followed a very similar pattern with the areas where
*Prootoceces* was most common corresponding to the region of the main
mussel banks.

The high density of the main mussel banks and their total area
meant that the total number of potential intermediate hosts between
Chalkwall Bowling Green (site 5) and Clifftown (site 7) was colossal in excess of ten million. This figure of the total number of *Mytilus* occurring between site 5 and site 7 was based upon the estimate that there was 1 kilometre of *Mytilus* beds between the two sites and that the average width of the bed was about 50 metres. Taking a density of 200 *Mytilus* per square metre, the total population was equal to

\[1,000 \times 50 \times 200 = 10,000,000\]

This was, if anything, a conservative estimate of the total population in the area as it did not take into account the very large number of *Mytilus* that occurred outside the main mussel banks, and also did not take into account the fact that the density of much of the beds was far in excess of the figure of 200 used in the calculation.

2. Examination of *Mytilus edulis* for larval stages

Method

Because of the enormous number of potential intermediate hosts the finding of larval stages proved difficult. The examination of 300 *Mytilus* from an area in which the *S. plana* were heavily infected, as was carried out at an early stage of the research programme, may in point of fact only represent the number of *Mytilus* that would occur in \(\frac{1}{3}\) square metre.

The finding that they were all uninfected obviously did not mean that
a sample of Mytilus collected from a short distance away would not be heavily infected. It is quite possible that, due to tidal currents carrying the eggs of Protooeoes into certain areas, that loci of infection did exist.

Because of the extremely large numbers of potential intermediate hosts involved, the time consuming examination of large samples was generally superseded by the use of a rapid method.

Uzmann (1953) commented that infection of M. edulis by sporocysts of Protooeoes lead to a suppression of gametogenesis or complete atrophy of the reproductive organs. For this reason the 'rapid technique' consisted of opening and examining samples of Mytilus actually on the shore and discarding all but the specimens that lacked gonads or looked 'sickly'. In this way relatively large numbers of Mytilus could be examined rapidly, with the possibly infected Mytilus being retained for further examination back at the laboratory. The subsequent teasing apart of the Mytilus revealed whether they were infected.

Results

Of fifty five Mytilus edulis examined in this manner from Clifftown (site 7), five were found to lack gonads. The subsequent examination of these five Mytilus revealed that one contained structures,
in the blood sinuses and in the tissues between the rays of the foot protractor muscles, that were extremely similar to the sporocysts of Protopoeces as described by Usman (1953) and Stunkard and Usman (1959).

The subsequent examinations, at various times, of 426 Mytilus in the manner described and of 844 Mytilus collected and not examined until they were returned to the laboratory, only yielded one more Mytilus infected with these sporocysts. This Mytilus was one of a sample of 147 collected from Chalkwell Bowling Green (site 5).

Description of the sporocyst

The following description is based upon sporocysts recovered from dissected Mytilus, and studied alive.

The sporocysts were simple, saucate and, in all those stages found up to the present, pale yellow. The specimens so far recovered all contained undifferentiated germ balls inside, what appeared to be, daughter sporocysts (figs. 13 and 14). These daughter sporocysts 'crawled away' when the mother sporocyst was squashed (fig. 14).

Approximately fifty of these sporocysts were recovered from the single infected Mytilus. The sporocysts varied in size from 0.5 mm - 1.8 mm with a mean length of the twenty measured of approximately 1.0 mm.

The most immediately recognisable feature of these sporocysts was
Fig. 13. Sporocyst from *Mytilus edulis* collected from Clifftown, (site 7), 28.8.1969.
Fig. 14. Daughter sporocysts containing undifferentiated germ balls.
their extreme mobility. They were clearly seen to have transverse striations and were able to contract and change shape rapidly (fig. 15). The manner of movement of these sporocysts corresponded well to the description of the appearance and movement of the sporocysts of Cercaria brachiodontis, as described by Hopkins (1954). He stated that the "sporocysts looked and moved like active fly maggots".

The undifferentiated sporocysts were found in August and December of 1969 and it is worth noting that Uzmann (1954) stated that the undifferentiated sporocyst stages of Prooctoesca from Mytilus in America were dominant from July to October after which they gave rise to cercarial generations. Unfortunately, no such cercarial generations have yet been discovered although 406 Mytilus were examined in the early months of 1970.

Discussion

The two Mytilus edulis that were discovered to be infected, both lacked gonads and were in poor condition. The tissue of the infected Mytilus from Cliftstown was very bright orange in colour and the infected Mytilus from Chalkwell had numerous nematodes and ciliates inside its mantle cavity.

The fact that only two specimens of Mytilus infected with this
Fig. 15. Sporocysts from Mytilus edulis.

The photographs were taken with a time interval of three seconds to demonstrate the ability of the sporocyst to contract and alter shape.
Figure 15

[Image of microorganisms with a scale bar indicating 0.1 mm.]
sporocyst were recovered from over 1,000 examined would suggest an extremely low percentage infection of the population but, as had already been stated, it is very possible that loci of infection did exist. It is interesting that Stunkard and Uzmann (1959) recorded that only 0.5% of the Mytilus edulis at Woods Hole, Mass., were infected with Protozoa.

The main point in question is whether the sporocysts discovered in Mytilus from Clifftown and Chalkwell were the sporocysts of Protozoa. Certainly the sporocysts recovered from Mytilus in this region correspond exactly to the descriptions of Uzmann (1953), Hopkins (1954) and Stunkard and Uzmann (1959) with regard to morphological detail and type of movement. The finding of these sporocysts also corresponds well with the ecological data. Further evidence would be to state that, although much work has been carried out upon the parasites of Mytilus and other shellfish (see in particular Lebour, 1911; Bowers, 1965 and Cheng, 1967, for general literature) no sporocyst that in any way resembles the one discovered from M. edulis in the Thames Estuary has been described from this, or from any other, lamellibranch from anywhere in Britain. Unfortunately, because of a single case, this statement is not possible.
The one case is the description by Cole (1935) of a sporocyst and cercaria of unknown taxonomic position from *Mytilus edulis* collected from the estuary of the River Conway in North Wales. He described this sporocyst under the name of *Cercaria tenuana* and his description and drawings show remarkable similarities to the sporocysts found by the present author and to the sporocysts and cercariae of members of the sub-family Haplocladininae as described by the American workers (see Page 75). Uzmann (1953) in his paper on the sporocysts of *Protoceca* in *Mytilus* in America mentions the finding of Cole (1935) but declines to say more than that the orange colour imparted to the mantle lobes of infected mussels by orange pigmented sporocysts in *Cercaria tenuana* was characteristic of the infection in America.

"Orange sickness" of *Mytilus* was described by Cole (1935) from one infected specimen of 1,000 *Mytilus* examined. He stated that previous to this examination six infected *Mytilus* had been discovered from 300 - 400 examined.

Cole (1935) described the sporocyst as being oval, moderately contractile and varying considerably in size, with average dimensions of about 1.1 x 0.45 mm (c.f. Uzmann, 1953; average length of sporocyst of *Protoceca* was 1.2 mm. Present work was approximately 1.0 mm).
The sporocysts were very thin walled with the tail-less cercariae being clearly visible through the walls.

Significant again is Cole’s comment that the whole sporocyst had a constant wriggling motion with the anterior end being in a constant explorative motion, “rather resembling a turbellarian”, (c.f. Hopkins, 1954, “... orange - pigmented sporocysts which looked and moved like active fly-maggots”). Cole (1935) also discovered early sporocysts which contained either a densely granular fluid or round masses of cells.

The description of the sporocyst that Cole found has similarities to the sporocyst found by the present author and significantly to the sporocysts described by Hopkins (1954) and Uzmann (1953), the latter of which was demonstrated to be the sporocyst of Proctocephes.

Although no cercariae have been found by the present author, the comparison of the cercariae described by Cole (1935) to those described by other workers bears examination.

The cercaria described by Cole (1935) was tail-less. Cable (1954) lists four main families in which the cercariae of some of the members have lost their tails or it is merely a knob of cells. These families are the Fellodistomatidae, the Brachylaemidae, the Microphallidae and the Hennorchidae. To these may be added the Zoogonidae.
The Brachylaemidae can be immediately discounted from this discussion on the basis that their larvae parasitise land and freshwater snails.

The Monorchidae and the Zoogonidae can also be excluded from this discussion because, unlike *G. tenuane*, their larvae are spinous and have the ventral sucker in the anterior half of the body. There are also other morphological differences including the fact that the cercariae of the Zoogonidae possess a stylet.

The cercariae of the Microphallidae are spinous and small suckered. According to Dawes (1946) and Yamaguti (1958) the Gymnophallinae is a sub-family of this group. Cable (1953) presents convincing evidence that the Gymnophallinae is best considered as a sub-family of the Family Peltodistomatidae. This classification and the taxonomic characteristics on which it is based is followed in this thesis.

The cercariae of the Gymnophallinae, although usually spinous and small suckered, have characteristics that are also apparent in the cercaria described by Cole (1935). The main similarity is in the extent of the digestive caecae. For this reason the sub-family Gymnophallinae must be considered as a possible taxonomic location for *G. tenuane*. 
Another sub-family of the Peltodistomatidae, the Haplocladinæ, is also typified by its tail-less cercariae. They are similar to _C. tenuens_ by possessing spineless integuments, and having a large ventral sucker in the posterior half of the body. The cercariae described for _Proctoeces_ by Uzmann (1953) and by Stunkard and Uzmann (1959) and _Cercaria brachiodontis_ as described by Hopkins (1954) have been placed within this sub-family.

It thus appears from basic identification that the tail-less cercaria described by Cole (1935) could possibly be included in the family Peltodistomatidae, possibly as either a member of the sub-family Gynophallinæ or the sub-family Haplocladinæ. A comparison of _C. tenuens_ with the cercaria described by Uzmann (1953) and with the cercaria described by Hopkins (1954) is carried out below to demonstrate similarities with these members of the sub-family Haplocladinæ.

The dimensions of each of the three cercariae are listed below.

Comparisons of this type never take into account differences that could be accounted for by different degrees of squashing or the effects of fixatives. It must be remembered that the differences between _C. milfordensis_ (Uzmann 1953) and _C. brachiodontis_ (Hopkins 1954) were insufficient for Stunkard and Uzmann (1959) to alter their opinion that
the two were very closely related. Further comparison is afforded by the illustrations (Fig. 16) of each of the cercariae and of a juvenile Prootoeceae maculatus (Stunkard and Ussmann, 1959).

<table>
<thead>
<tr>
<th></th>
<th>C. tenuans</th>
<th>C. milfordensis</th>
<th>C. brachiodontis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cole (1935)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ussmann (1953)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hopkins (1954)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mean body length (mm)</td>
<td>0.3</td>
<td>0.254</td>
<td>0.33 - 0.43</td>
</tr>
<tr>
<td>Oral sucker diameter (mm)</td>
<td>0.05</td>
<td>0.043</td>
<td>0.075</td>
</tr>
<tr>
<td>Ventral sucker diameter (mm)</td>
<td>0.07</td>
<td>0.048</td>
<td>0.09</td>
</tr>
<tr>
<td>Distance from anterior end to centre of vent. sucker</td>
<td>0.015</td>
<td>(from illustr.) Ventral sucker is posterior to middle of body</td>
<td></td>
</tr>
</tbody>
</table>

It can be seen from the table that the dimensions of C. tenuans are similar to those of C. brachiodontis and C. milfordensis. Not only is the ventral sucker larger than the oral sucker in all three, but it is located in a similar area of the body. As already stated, gymnophallids invariably have small ventral suckers.

A prepharynx is described by all three authors for their respective cercariae and each has a distinct pharynx. This is a feature found in both members of the Gymnophallinae and the Naplocladinae. The shortness of the intestinal caeca in C. tenuans is a feature more in
Key to figure 16. A comparison of the cercariae and juvenile Proctoeca.

DC  Digestive caeca
ES  Excretory system
OS  Oral sucker
P   Pharynx
T   Testis
VS  Ventral sucker
Fig. 16. Comparison of cercariae and juvenile Proctoeces.

Cercaria milfordensis
(Uzmann, 1953)

Cercaria brachiodontis
(Hopkins, 1954)

Cercaria tenuana
(Cole, 1935)

Proctoeces maculatus
(Stunkard and Uzmann, 1959)
accord with members of the Gymnophallinae although Cable (1953) states that the intestinal caeca do terminate anterior to the ventral sucker in some members of the Haplocladinidae. The excretory bladder in C. tenuans, with its two arms extending anteriorly, is similar to the very characteristic Y-shaped excretory system of C. milfordensis, C. brachiodontis and the adult Proctocea.

As shown by comparison of the illustration of C. tenuans with a juvenile Proctocea (fig. 16), other features are consistent with the organisation of the adult Proctocea. Particularly noticeable is the tandem arrangement of the testes in both. Cole (1935) also describes the developing cirrus sac of C. tenuans as opening anterior to the ventral sucker, a feature found in the adult Proctocea. Both these can be considered as characteristic of members of the Haplocladinidae but according to Cable (1953) they are not in accord with the characteristics of the Gymnophallino. It is interesting that Cole (1935) was able to observe this development within a cercaria just released from a sporocyst.

On the basis of the evidence presented it thus appears that C. tenuans has many similarities to the cercaria described for members of the family Fellodistomatidae. Although not in complete accord, it
appears to possess more features characteristic of members of the sub-family Haplocladininae than with members of the Gynnophallinae. For this reason it is tentatively suggested that C. tenuans, as described by Cole (1935), could possibly be the larval stage of a member of the sub-family Haplocladininae, of the family Fellodistomatidae.

This description of a larval digenean by Cole (1935) has been considered in such detail because of its importance with regard to the present discovery. Although a comparison between the sporocyst found from two Mytilus in the Thames Estuary with the schematic drawing and description of a sporocyst found by Cole (1935) must be inconclusive, the fact that a similar sporocyst has been described from the same host means that the descriptions must be considered carefully. If evidence was available that C. tenuans was the larval stage of a trematode, unrelated to the Fellodistomatidae, then it would cast serious doubt upon the significance of the present discovery in the Thames Estuary. As it has been demonstrated that C. tenuans could possibly be the larval stage of a member of the same, or a closely related sub-family of the Fellodistomatidae as Prostostomes, then it is considered that its discovery, from an area far removed from the present study, in no way alters the suggestion that the sporocysts found in M. edulis in the
Thames Estuary could be those of Protoceace. Only the finding of more examples of the sporocyst from the Thames Estuary and the establishment of the life cycle of Protoceace under controlled conditions will verify the hypothesis.

C. tenuans, to the author's knowledge, has only been recorded once in the literature since 1935, although much work has been carried out upon the parasites of Mytilus and other shellfish (see Cheng, 1967, for literature). This discovery was by Figueirdeo et al (1964). They described the sporocyst from M. edulis collected on the coast of Portugal and they also noted its great mobility.

It thus appears possible that C. tenuans as a parasite of M. edulis is fairly uncommon. The finding of Protoceace from Buccinum undatum from Millport, in Scotland, (material given to the author by R. Wooton) suggests that this trematode may be more common around the coast of Britain than was at once envisaged. It further suggests that larval stages of Protoceace may be found in areas from which adults have not been reported. In this context it is interesting to note that Buccinum undatum is known to feed upon Mytilus edulis.
3. Suggested life cycle, on the basis that Mytilus edulis is the

first intermediate host

Because of the reasons that have been discussed previously (P71-2) it is suggested that no fish host is involved in the life cycle of Protocees in the Thames Estuary and that the life cycle involves two lamellibranch molluscs. The life cycle suggested below, and illustrated in Fig. 17, can only be a hypothesis until infection experiments are carried out under controlled conditions, and the involvement of Mytilus edulis is confirmed or repudiated.

Adult Protocees in the kidney of S. Plana produce eggs which contain motile, ciliated miracidia. These eggs are released into the kidney of the S. Plana and from here pass, via the renal pore, into the supra-branchial space of the gills. From here they are expelled via the exhalant siphon.

It is not known what happens to these eggs once they have been expelled. Freeman and Llewellyn (1958) could not observe an operculum to the egg and could not induce release of the miracidium.

Dolgyok (1967), in describing a sexually mature Protocees from the prosobranch, Rissoa splendidia from Russia, describes an operculum to the egg. Loch-Frank (1969) states that the operculum of the eggs of Protocees from S. Plana from Germany are visible only after removal
Fig. 17. Suggested life cycle of Proctoeces subtenuis in the Thames Estuary.
of the miracidium. She does not describe how this removal of the
miracidium was achieved but states that the miracidia move a short
distance and then die.

It is not known at what stage, under natural conditions, that
the miracidium is released from the egg. It is possible that the
complete egg would be taken in by *Mytilus edulis* during normal filter
feeding. Certainly the size of the egg: $0.042 \times 0.024 \text{ mm}$ (Freeman
and Llewellyn, 1958) is consistent with the size of particles retained
by *M. edulis* during suspension feeding, (Jørgensen, 1966). Once in
the gut of the *Mytilus* the egg shell could be digested and the
miracidium released. The placing of fertile eggs in *Mytilus juice*
by the present author, did not appear to induce hatching although
this is of course not conclusive as many other factors could be involved.

It is possible that the miracidium is released from the egg
before it reaches the first intermediate host and that the miracidium
then carries out its usual role in the life cycle of digeneans by
searching out the first intermediate host.

By whatever manner the first intermediate host was successfully
infected, the result would be the development of a sporocyst generation
in the sinus system of the *Mytilus*. These sporocysts would give rise
to daughter sporocysts and these would develop in the sinuses and
interfollicular gonad of the *Mytilus* and cause cessation of gameto-
genesis and atrophy of the gonads.

In early spring these sporocysts would give rise to microcercous
cercariae that would be released from the *Mytilus*. Uzmann (1953)
demonstrated that the survival of *Protoeces* cercariae could be quite
sustained: In filtered sea water at 7°C there were no mortalities
after seven days, whilst about 30% were still alive after twelve days.

Uzmann (1953) suggested that this long survival might partially
compensate for the lack of natatory ability displayed by these cercariae.

The only activity of these microcercous cercariae after emergence,
that Uzmann (1953) could detect, consisted of a periodic contraction
and elongation which effected a serpentine, but random, progress
across the substratum. He was also unable to detect any pronounced
taxes of these larvae and suggested that their role in completing
the life cycle appeared to be decidedly passive in contrast to the
host seeking activity of many other cercariae.

This means that a close association of the intermediate and final
hosts would be vital for a reasonable chance of infection. If the
two populations of the hosts were separated it would rely mainly upon
tidal currents to carry the larvae into the vicinity of the *S. plana*; the greater the separation, the less the chance of the cercariae reaching the region of the *S. plana*.

Once in the region of the *S. plana*, the cercariae would rely on being 'sucked up' by the inhalant siphon of a final host. The fact that locomotion of the cercaria is confined to crawling across the surface of the substrate could be one of the reasons why the adult is found within a surface deposit feeder such as *S. plana*.

Once ingested by the *S. plana* the cercaria could possibly reach the kidney region in one of at least, three ways: It would have to pass through a gill lamella and then make its way to the suprabranchial space. From here it would have to go 'against the flow' in order to reach the kidney by going through the renal port. This passage from the suprabranchial space to the kidney would thus be the journey that anything leaving the kidney would make, but in reverse.

The other two methods of entry into the kidney would necessitate the cercaria having to bore its way through tissue. Although not possessing stylet, both Ussmann (1953) and Hopkins (1954) describe their respective cercaria as possessing cephalic glands, which in the case of *Cercaria milfordensis* terminate in six separate pores on
the dorsal lip of the oral sucker. The secretions of these glands might assist entry into the tissue of the final host.

In the case of Proclec s in the Thames Estuary, if present, they could assist the cercaria to bore through the tissue in the region of the kidney and so obtain direct entry into the preferred region.

If the cercaria is taken into the gut of S. plana it will eventually be carried towards the kidney region. As depicted on page 22, the hind gut actually passes through the heart which is situated in the pericardium. Reference to the anatomy of S. plana will demonstrate that if the cercaria bored out of the intestine in this region it would have a very short journey to the kidney.

Which of these three methods of entry is correct, if any, is unknown, as is the nature of the stimulus that attracts the cercaria to the kidney region of S. plana. It is suggested that some stimulus would be necessary as the cercaria would not be carried passively into this region by the actions of the host.

Once within the kidney region of the host the cercaria would have to ensure that it remains there and is not passed out through the renal pore. The life cycle would be completed when the cercaria became sexually mature.
Summary:

From an investigation of the distribution of prosobranch and lamellibranch molluscs along the north coast of the Thames Estuary it was concluded that *Mytilus edulis* was the possible intermediate host of *Protoechoes* in this area. The examination of numbers of this lamellibranch was rewarded by the discovery of two specimens infected by sporocysts which were very similar to those described by American workers for *Protoechoes*.

It is considered that it is probable that the sporocysts found within *M. edulis* from the Thames Estuary do represent the larval stages of *Protoechoes* in this area. The description of *C. tenuans* by Cole (1935) does not necessarily modify this hypothesis although only the finding of more examples of the sporocyst from the Thames Estuary and the establishment of the life cycle of *Protoechoes* under controlled conditions will verify the hypothesis.

On the assumption that *M. edulis* is the intermediate host of *Protoechoes* in the Thames Estuary a life cycle has been suggested.
CHAPTER V  GENERAL CONSIDERATIONS REGARDING THE LIFE HISTORY  OF PROCTOEDES IN THE THAMES ESTUARY

Much of the following discussions are based upon generalisations that would apply whatever the identity of the first intermediate host, but part is based upon the assumption that M. edulis is involved in the life cycle of Proctoeces in the Thames Estuary. Because this has not been proved conclusively, this part could be considered unjustified. The fact that hypotheses involving M. edulis fit the known facts well, is considered as further evidence of the plausibility of the suggestion.

1. Localisation of the endemic areas of Proctoeces

As has already been stated, the lack of natatory ability of the microcercous cercaria could account for the localisation of the parasite in S. plana to regions where the first intermediate host is present. This, coupled with the apparent lack of any host seeking ability by the cercaria, would most probably mean that the chance of any cercaria infecting a S. plana would be slight if it was not released directly into the vicinity of the S. plana. Other cercariae may be carried by the tidal currents out of the immediate vicinity of the final host. A proportion could possibly be carried into other areas of S. plana and this could explain the rare occurrence at Whitstable, as discovered by Freeman and Llewellyn (1958). If, as at Whitstable, there was a
population of *Mytilus* than the complete life cycle could become established in this new area.

The rare occurrence of *Protooeaes* at Benfleet Bridge (site 1) and at Leigh Bridge (site 3) could also be accounted for by suggesting that the tidal currents had carried cercariae from the *Mytilus* population at Benfleet Creek (site 2) into these areas of *S. plana*.

This lack of natatory power of this microcercous cercaria is most probably the main reason why the parasite has apparently not spread to the Kent coast or to the neighbouring rivers of the Crouch and Blackwater. Tidal flow would also most probably mean that there is no mixing of water from the north coast of the Thames Estuary and the South coast directly opposite, and therefore most probably no cercariae would ever reach the area. Even if they did, the scarcity of *Mytilus edulis* would prevent a successful continuance of the life cycle.

It is interesting that this localisation of the endemic areas of *Protooeaes* observed to occur in the Thames Estuary was similarly noted by Ussmann (1953) to occur in America. He commented that the endemic areas of *Protooeaes* were quite localised with respect to each other and that sites on the north/south reaches of Long Island Sound bore infected
Mytilus, whereas those sites on the other side, where ecologically, the intertidal areas were practically identical, were uninfected. So here again there is the situation where seemingly a channel of water prevents the spread of the parasite.

If Mytilus edulis is the intermediate host for Protoceoes in the Thames Estuary region, it may be that Mytilids are generally the first intermediate hosts for Protoceoes. Yamaguti (1938) described a larval trematode, that he assigned to the genus Protoceoes, from the Mytilid Brachiodontis senhausi. Ummann (1953) and later Stunkard and Ummann (1959) described the larval and adult stages of Protoceoes from Mytilus edulis in America. Hopkins (1954) description of a larval Fellodistomatid that Stunkard and Ummann (1959) considered to be very similar to the cercaria of Protoceoes, was based on the sporocysts and cercariae recovered from the Mytilid Brachiodontis recurvus, again from America.

As stated by Wright (1960) most digenetic flukes that have been investigated have been found to exhibit a more marked degree of host-restriction in their larval forms than their adult forms. If this generalisation is true for Protoceoes it will be interesting in the future to see if the larval stages of adult Protoceoes from fish and, more particularly, from invertebrates are found in Mytilids.
In this connection it is interesting to speculate again upon the possible location of the larval stages of adult Protoceces that have been found by Loos–Frank (1969) and by Wooton, (pers. comm.) from the Whelk, Buccinum undatum. It has been suggested previously (page 76) that the high level of infection of individual Whelks in both cases could be due to the whelks in question feeding upon an infected first intermediate host and thus obtaining a large number of cercariae. It is interesting that Mytilus edulis is listed by Fretter and Graham (1962) as being one of the lamellibranchs commonly eaten by Buccinum undatum, and so it is conceivable, although there is no direct evidence to suggest it, that Mytilus is involved again. An examination of Buccinum from areas around the British Isles for Protoceces could prove interesting.

2. Introduction of Protoceces into Britain.

Freeman and Llewellyn (1958) suggested that the limitation of Protoceces to the region of the Thames Estuary, in a lamellibranch widely distributed around the British Isles, strongly suggested that the introduction of P. subtenuis to Britain was recent. They further suggested that the introduction could have most probably occurred by the accidental introduction of the first intermediate host.
Loos-Frank (1969) considered that the existence of *Proctoeoes* as a sexually mature form in *S. plana* in the North Sea could be due to the fact that the previously infected fish hosts had had to move away, possibly because of climatic changes, and the parasites then could only survive if they could adapt themselves to having a mollusc as a final host. This argument appears to assume a great deal for which there is no evidence. She is also mistaken in stating that no Labrids or Sparids occur in the North Sea (see page 71).

The occurrence of a sexually mature digenean in an invertebrate is, from an evolutionary point of view, primitive, although this is not to say that this condition in *Proctoeoes* is necessarily primitive. It is possible that members of the genus *Proctoeoes* are facultative parasites of fish and will become sexually mature in invertebrates. In this way, if a fish ingested a mollusc containing a sexually mature *Proctoeoes* the parasite might well survive for some period of time in the gut of the fish, as suggested by Stunkard and Ussmann (1959). This could explain the large number of records of members of the genus from many species of fish. The fact that *Proctoeoes* from *S. plana* are capable of surviving in the labrids *Crenilabrus melops* and *Crenilabrus rupestris* was demonstrated by Freeman (1962a).
It is possible, as suggested by Loos-Frank (1969) that there are species of Protozoa that need a fish final host, others that need an invertebrate and others that will utilise either. As the 'normal' location of Protozoa is not known it is very difficult to suggest possible reasons for its existence in the Thames Estuary.

If *P. subtenuis* is to be regarded as primarily a parasite of fish from tropical and sub-tropical seas then, as previously stated, its introduction into this country could have occurred by the accidental introduction of the first intermediate host.

*Mytilus edulis* and other *Mytilids*, are very common fouling organisms of ships (Clay 1965) and so the introduction of an infected *Mytilus* into any area where ships passed close by could be easily accomplished. This is obviously the case with the Thames Estuary.

*Mytilus edulis* has a world wide distribution in the northern hemisphere and so the introduction of *Mytilus* or other *Mytilids* into an area would go unnoticed. It also means that it would be very likely that there would be *Mytilus edulis* present to act as first intermediate hosts should the life cycle become established.

In the case of the Thames Estuary it could be postulated that some infected *Mytilus* or other *Mytilids*, were brought into this area
from overseas, possibly attached to a ship. Some of the larval stages of Prootoecea, once released, may have been subsequently 'sucked up' by the inhalant siphons of Serobicularia. For some reason they were not only able to survive in the kidney region of this mollusc but actually became sexually mature and produced fertile eggs which were passed out of the exhalant siphon of the Serobicularia. Indigenous Mytilus, being common in the area, allowed for the continuance of the life cycle.

This suggestion of the way in which Prootoecea may have been introduced into the Thames Estuary, takes account of the known facts and fits the hypothesis that M. edulis is involved in the life cycle. Unfortunately this latter assumption is unproved.

Whether Prootoecea are truly parasites of vertebrates or invertebrates and also the exact locality from which introduced first intermediate hosts may have originated is, at least at this juncture, impossible to ascertain.

3. Abbreviated life cycles and the phenomenon of progenesis

That abbreviated life cycles can occur in digenetic trematodes has been demonstrated experimentally by, among others, Buttner (1955) for Plagiorhia brumpti, Razaia joyeuxi and Paraspodermat brumpti and...
by Stunkard (1959) for Asyaphyloclora amnicola.

The finding of all developmental stages from sporocysts to gravid adults of Proctoeces within Mytilus edulis in America by Stunkard and Uzann (1959) demonstrated that the life cycle of Proctoeces can be restricted to invertebrates in American waters. This discovery and the evidence for abbreviated life cycles in trematodes means that the suggestion that the life cycle of Proctoeces in the Thames Estuary involves two lamellibranchs is not entirely without precedent.

There seems no reason not to regard the sexually mature Proctoeces found within S. plana in the Thames Estuary as an adult trematode. All the evidence suggests that a fish host is not involved in the life cycle and so the Proctoeces in the mollusc is the stage that brings about a continuance of the life cycle. Consistent with this view is the fact that the Proctoeces in the mollusc can produce fertile eggs that contain motile, ciliated miracidia and these are passed out from the host. As Freeman (1962a) showed, Proctoeces (obtained from S. plana) that had been in a fish host for a period showed no difference to the Proctoeces obtained directly from the mollusc. Thus there is no evidence for any maturation of the worm whilst in a
fish host. It therefore appears that the trematodes in *S. plana*
are directly comparable, structurally, functionally and in their
status in the life history with the adults found in the hind gut of
fishes in other parts of the world. There thus seems no reason to
modify the emphasis on their adult nature by invoking the phenomenon
of progenesis.

The term progenesis was first used by Giard and Bonnier (1887)
to describe the assumption of sexual maturity by male parasitic isopods
while still in the larval condition; while in a state of arrested
development. The term "méta-cercariae" was proposed by Dollfus (1913)
to designate the immediate post cercarial stage of trematodes, whether
encysted or not. It was also Dollfus (1924) who first applied the
term progenetic to gravid metacercariae of digenetic trematodes.

Difficulty arises because it is not always possible to
distinguish metacercariae from adults. For this reason the term
progenesis has been used by several authors, for example Janieson
(1966 a & b) to describe the occurrence of sexually mature trematodes,
irrespective of their state of development, in non-definitive hosts.

This, of course, presupposes that the author knows the definitive
host and that he can state with certainty that the invertebrate is not
the normal final host and the vertebrate only facultative, as it could possibly be in some species of Proctoeces. Thus in many cases the term progenesis has been used in the literature on trematodes in a different context to which it was originally intended.

For this reason the present author considers that it is not necessary to invoke its use when discussing Proctoeces from S. plana from the Thames Estuary. This might equally well apply to the other records of sexually mature Proctoeces occurring within invertebrates from Russia, U.S.A., Germany, Japan and Morocco. To define these as progenetic simply because they occur in an invertebrate appears to the author to be a mis-use of the term. The fact that the evidence strongly suggests that the complete life cycle of Proctoeces from Mytilus in America and of Proctoeces from S. plana in Britain only involves invertebrates further suggests that these trematodes are adults in every sense of the word.

The present author thus considers that if a trematode is sexually mature and brings about the continuance of the life history and has all the morphological characteristics of an adult, there is no reason not to regard it as such, even if it has been found within an invertebrate.
The occurrence of an adult trematode within a mollusc and the complete life history within lamellibranchs is surely, at least, representative of a primitive condition. It is possible that the type of life history exhibited by Proctoeces in S. plana is similar to that which the early flukes may have gone through. The involvement of a vertebrate into the life cycle may have been due to an extension of the food chain. If, as shown by Freeman (1962a) for Proctoeces from S. plana, the trematode was able to survive in the fish then here was the possible beginning of the involvement of a vertebrate in the life cycle. During evolution the deferment of sexual maturity until the worm was in the vertebrate host would then bring about a similar situation to that exhibited by the majority of the present day digonatia trematode parasites of fish.

The present author does not intend to discuss here the evolution of the trematoda or the evolution of the various larval stages but it is interesting to consider, on the basis of the suggested life cycle of Proctoeces in the Thames Estuary, the advantages or disadvantages of having a vertebrate in the life cycle.

As pointed out by Wright (1960) the geographical range of a parasite is limited by the range of its hosts, its effective range.
being limited by the concurrence of all hosts so that the life cycle can be completed. As he also points out the ecological requirements of most molluscs are such that a species is seldom uniformly distributed throughout its range but is broken up into a number of populations between which there are varying degrees of isolation.

Both these points are well illustrated by Prootoeoes in the Thames Estuary. Only where S. plana and Mytilus edulis occur together is the life cycle of Prootoeoes found to be successful. The lack of S. plana and/or M. edulis in certain areas, coupled with the apparent lack of dispersal power of the cercaria, has contributed to the lack of spread of Prootoeoes and the isolation of the parasite to the one locality.

This is a point in favour of the inclusion of a motile vertebrate in the life cycle. As Wright (1960) points out, if the final (vertebrate) host ranges widely, carrying the egg-laying adult trematode in its body, then there is a great possibility that eggs will be passed in an area where they can infect a different mollusc population. If in the case of Prootoeoes in the Thames Estuary a fish host were involved it would most probably migrate throughout the general area of the Thames Estuary and thus possibly spread Prootoeoes to other areas where S. plana and M. edulis occur.
Jamieson (1966 a & b) considers that the dispersive advantage of having a vertebrate in the life cycle may have been the major factor leading to the usual deferment of sexual reproduction to a non-molluscan host.

The fact that a vertebrate is mobile, widely dispersed and is more resistant to changes in the environment means it is less affected by adverse conditions. Such conditions might severely reduce or even eliminate a population of invertebrates, and with it the parasite could be eliminated. Protoeces as a parasite of S. plana in the Thames Estuary is an example of this possibility. The severe winter of 1969/3 severely reduced the numbers of many molluscs, including S. plana and M. edulis. With Protoeces having such a limited distribution, the elimination of these molluscs from this one area might have caused the elimination of the parasite. The incidence of infection in a fish host, even if very low, would nevertheless be advantageous as a reservoir for the infection of the lamellibranchs in the event of a natural disaster reducing or eliminating the lamellibranch population. It is very unlikely that such a reservoir of Protoeces exists in a fish host in the Thames Estuary.

The suggestion of Stunkard (1959) that a vertebrate host is longer
lived and thus would prolong the life of a parasite is not altogether consistent with the evidence. As stated by Wright (1960) the life of some adult flukes is very short. This is also suggested for Proctoeces by the experimental infection of Wrasse with Proctoeces by Freeman (1962a). He found that only about 5% of the introduced parasites remained after about five days and that in Crenilabrus rupestris all were lost by six days. On one occasion five parasites, out of about 250 introduced, remained in C. molora for about 12 - 15 days. This suggestion that the life of adult P. subtenuis is not long in a fish is largely consistent with the information available in the literature.

Manter (1947) found only six specimens of P. subtenuis from four of twenty nine hosts examined at Tortugas, and Manter and Pritchard (1962) found four specimens from two of forty nine hosts from Hawaii. Rees (1970) found only a single specimen from one of eight fish from Bermuda. The majority of the other records in the literature do not state what number of hosts were examined, but in most cases only one or two P. subtenuis were recovered. This evidence could suggest that the life of the adult Proctoeces in a fish is short and thus at any one time only a small percentage of the fish population are infected. It could be equally well be taken as evidence that the life cycle of
Proctoeces is not adapted to a fish final host and possibly even that the adult Proctoeces is truly a parasite of molluscs and only a facultative parasite of fish, surviving for a short time in the fish after the mollusc is eaten.

Jennison (1966) would accept the fact that the occurrence of an adult fluke in a fish is rare as compared with its occurrence in an invertebrate, as evidence that molluscs may offer an environment superior to that afforded by a fish for the development of flukes to maturity. He appears to base this contention on the fact that flukes of a given species are rarely numerous within a fish host. Certainly a comparison between the number of Proctoeces in S. plana at Clifftown, (where over 90% are infected with an average of between four and five worms) and the numbers of Proctoeces recovered from fish might be taken to enhance his hypothesis, although many other factors, such as the method of infection of the final host have to be taken into account.

Clearly the utilisation of non-molluscan hosts nevertheless has been favoured by selection as it appears to be the normal condition in the Digenea. The dispersive advantage and the fact that vertebrates are less likely to be affected as a population by adverse conditions, may have been major factors leading to the deferment of sexual reproduction to a non-molluscan host.
SECTION II
CHAPTER VI

OSMOTIC RESPONSES OF THE KIDNEY FLUID OF SCROBICULARIA PLANA

Estuarine lamellibranchs that have been studied appear to exhibit little or no osmoregulatory ability. _Mya arenaria_ and _Mytilus edulis_, for example, can be found at salinities of as little as 4-5%, but still the osmotic pressure of the haemolymph conforms to that of the environment (Schlieper, 1955 and 1957). Potts (1954) demonstrated that the blood of _M. edulis_ was isosmotic (-0.02°C) with the external medium from a depression of freezing point (Δ) of 2.09°C to 0.58°C.

Another mytilid, _Mactra demissus_ has been recently studied by Lent (1969). This euryhaline species has a lower limit of salinity of 5%. Experiments equilibrating mussels with 10, 15, 20, 30, 40 and 45% for three days and then determining the depression of freezing point showed that _Mactra demissus_ was isosmotic between 9 and 43%.

The work of Cole (1940) on _Venus (Mercenaria) mercenaria_ is sometimes quoted by authors (Hopkins, 1949; Nicot, 1967) as an example of a bivalve that shows an ability to maintain itself hyperosmotic in dilute sea water. Cole (1940) demonstrated that the water in which the clams occurred had a depression of freezing point of 1.336°C, whereas the mantle cavity fluid of the clam was Δ1.369°C and the blood was Δ1.386°C.
Although no information is presented to show when the samples were collected, it appears that these figures were obtained for fluids taken from the animals soon after collection, and these were compared with the external medium estimations made at some undetermined time. No equilibration with known salinity external media for substantial periods is mentioned and so the surprising difference between the mantle cavity fluid (usually only sea water trapped between the valves when the animal closes) and the external sea water could be explained on the basis of a time difference in the two estimations. The animals may well have closed at a time when they were inundated with higher salinity sea water with which they had come into equilibrium. This is further suggested by the fact that the $\Delta$ of blood was only 0.017°C different from the $\Delta$ of the trapped mantle cavity fluid. The collection of clams for analysis of the body fluids at a later time (for example, at low water) when the salinity of the external medium may well have altered, could possibly account for the observed 0.05°C difference in freezing point depression between the external medium and the blood.

That this is a possibility is illustrated by Milne (1940) who recorded that the salinity inside the mantle cavity of Mytilus at low
tide was 24% when the salinity outside had fallen to 7%. It would therefore appear likely that *Venus (Mercenaria) mercenaria* conforms to the general picture of estuarine lamellibranchs by exhibiting little or no osmoregulatory ability.

The response of *S. plana* to osmotic pressure changes was first studied by Freeman and Rigler (1957) on animals collected from the mud flats at Chalkwell, in Essex. These authors demonstrated that the osmotic pressure of the blood of *S. plana* was not statistically different from that of the external medium down to about $\Delta 1.05^\circ C$, but that, after a period of 110 hours equilibration, the blood of specimens in dilute sea water ($\Delta 0.59^\circ C$) had a mean $\Delta$ of $0.74^\circ C$.

There is doubt expressed by Robertson (1964) whether the animals in the experiment were showing active control of the blood concentration at low salinities, or whether they had resisted final dilution to the outside level by keeping the valves of their shells closed.

This latter point is discussed in detail by Freeman and Rigler (1957). They demonstrated that short term changes in the salinity of the external environment may have no effect upon the osmotic pressure of the haemolymph of *S. plana* because of behavioural reactions that prevent the animal from coming into contact with adverse salinities.
Thus a change in the total osmotic pressure of the external medium would cause closed animals to remain closed and cause open animals to retract their siphons and close their valves. Thus in both cases the animals would protect their blood from extreme changes in concentration. The assessment of the outside conditions by closed animals most likely occurs via the free edges of the mantle lobes which retain contact with the external medium, even when the valves are closed.

Freeman and Bigler (1957) also demonstrated that upon exposure to external media of lowered osmotic pressure _S. plana_ shows a greater reluctance to open than in sea water, there being a longer period of closure the greater the decrease in osmotic pressure. Thus half the animals equilibrated for one week in 100% sea water and then transferred, in a closed condition, to 90% sea water opened in 45 minutes. The time for half of the closed animals transferred from 100% to 40% sea water to open was in excess of a day.

The rate of equilibration of _S. plana_ with the external medium is rapid once the animal is open. The same authors demonstrated that the osmotic pressure of the haemolymph of open animals, previously equilibrated to 100% sea water and then placed in 80% sea water,
reached 50% equilibrium in just over half an hour and, as closely as
they could measure it, complete equilibration in 4–5 hours.

Approximately the same half time was true for the more rapid change
in osmotic pressure of the blood of animals transferred from 100% to 60% sea water. In comparison, it was demonstrated that *S. plana*
transferred from 100% to 60%, and that remained closed, showed an
average drop in the osmotic pressure of the blood of only about 1.5%
per hour. This change presumably occurring via the free edges of the
mantle lobes.

Those facts concerning the reactions of *S. plana* when transferred
to dilute media and the times of equilibration demonstrated by
Freeman and Rigler (1957) have been considered in detail because of
the importance of allowing sufficient time for equilibration of *S. plana*
when the osmotic responses of its kidney fluid are to be investigated.

Investigations of the osmotic responses of estuarine bivalves
have always been carried out upon the blood of the animal.

Information on the physiology of lamellibranch kidneys is scarce.

Freeman and Llewellyn (1958) had shown that at 100% and 70% sea
water there was no significant difference in the osmotic pressure of
the tubular fluid of *S. plana* and that of the external medium. In order
that the environment of *P. subtenuis* might be defined more closely, the osmotic responses of the kidney fluid to changes in the concentration of the external medium were investigated.

**Materials and method**

Animals used in this series of experiments were collected from localities on the north coast of the Thames Estuary, in the vicinity of Southend-on-Sea. They were transported to Queen Mary College and there kept under conditions of constant temperature (for a day or less) before they were used for experimentation.

Groups of these *S. plana* were placed in sea water of various dilutions, obtained by dilution of the stock sea water with glass distilled water, and kept at 15±1°C. The stock sea water (average chlorinity = 18.76‰ which is equivalent to a salinity of 34.09‰) is referred to throughout as 100‰ sea water.

**Time of equilibration**

Because of the reasons discussed previously, the time of equilibration with the various dilutions is of the utmost importance. Reference to the data of Freeman and Higler (1957) - (cited page 133) - demonstrates that the equilibration times used in this series of experiments (minimum of three days in 100‰ and a minimum of seven days
in 30\% sea water) were in excess of that needed.

As a further precaution only animals that had been seen to have had their siphons extended, when inspected periodically during the previous 48 hours, were used for experimentation. Chapman and Newell (1956) observed that the inhalant siphon of *S. plana* was protruded soon after initial gaping of the shell valves and that the siphon was open at its tip during extension. The protrusion of the inhalant siphon therefore provides a convenient indication that the animal is open from the aspect of being exposed to the external medium.

**Method of analysis**

The osmotic pressure of the samples of the kidney fluid and of the external medium was determined by depression of freezing-point.

The apparatus used for the examination and collection of the samples was first described by Ramsey and Brown (1955) and the reader is referred to this paper for the description of the apparatus. Only modifications to the original apparatus and procedure are discussed below.

**Obtaining samples from the kidney**

After the period of equilibration the *S. plana* were opened and any mantle cavity water removed by gentle blotting so as to ensure no
contamination of the kidney fluid. The samples of the kidney fluid were rapidly drawn out of the kidney using a very fine glass pipette attached to a length of rubber tubing by which the operator could suck up a controlled amount of fluid. This operation was performed with the aid of a low power binocular microscope.

The fluid thus obtained was rapidly pipetted under liquid paraffin on a watch glass coated with a fine smear of silicone grease that provided a hydrophobic surface that prevented the drop of liquid from spreading out into a film. Leaving the sample under liquid paraffin prevented any alteration in the osmotic pressure of the sample due to evaporation.

The transference of small samples of this fluid (10^{-2} \text{mm}^3, \text{in this case}) was performed as described by Ramsey and Brown (1955).

**Modifications to the original apparatus**

On the present piece of apparatus the usual racking mechanism of the microscope was replaced by a modified micromanipulator. This had the advantage of not only allowing the usual focusing movement in a vertical plane but also for adjustment in the horizontal plane.

Because of this increased field of view it was found possible to have more samples in each specimen tube.
The original system for the illumination of the sample was found
to be inadequate and so in the present apparatus the light source was
an 8 volt, 48 watt projector bulb. The light was directed from this
via a piece of solid perspex with an attached mirrored surface. An
iris was incorporated into this system to allow for adjustment whilst
cross polaroids were found to be useful under certain circumstances.

The system for control of the temperature of the alcohol bath of
the apparatus was also different from the original design. The use
of dry ice and a simple system of two concentric vessels, both
partially filled with 90% alcohol, was superceded by the use of a
Camlab Thermostatic Refrigerator type TK1 cooling coil, which
allowed for easy and accurate temperature control. Once the
temperature of the bath had been reduced to below the expected
thawing point the cooling effect was reduced and the temperature
controlled by balancing the effect of the TK1 with that of the
heating wire.

With this system of temperature control it was possible to
 maintain the temperature of the bath constant to within 0.01°C for
periods in excess of 15 minutes. It was found convenient to leave
the two settings so adjusted whenever possible and make use of the
incorporated heater shorting switch to raise the temperature and the heater-off switch to lower it.

The sensitivity of this arrangement meant that it was possible to raise or lower the temperature by 0.002°C. By a routine of not quite allowing the last crystal to disappear, lowering the temperature and re-growing it, followed by very slow heating, it was possible to determine the thawing point of a sample to within 0.002°C. The reading of the final temperature on the Beckman thermometer was assisted by the use of a small magnifying lens.

**Calibration curve**

To calibrate the Beckman thermometer for use with the apparatus a series of standard sodium chloride solutions were used. These consisted of samples of 0.35 Molal, 0.30 Molal, 0.25 Molal and 0.20 Molal sodium chloride prepared with oven dried, Analar reagent.

The observed reading of the Beckman at the thawing point of these samples was then compared with the actual depression of freezing point for each of these molalities as obtained from the International Critical Tables.
The results of one such calibration are shown in table 7 and fig. 18.

**TABLE 7**

<table>
<thead>
<tr>
<th>Standard NaCl</th>
<th>Observed Beckman reading</th>
<th>Actual Depression of freezing point (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.35 Molar</td>
<td>1.380</td>
<td>1.184</td>
</tr>
<tr>
<td>0.30 Molar</td>
<td>1.215</td>
<td>1.017</td>
</tr>
<tr>
<td>0.25 Molar</td>
<td>1.038</td>
<td>0.850</td>
</tr>
<tr>
<td>0.20 Molar</td>
<td>0.875</td>
<td>0.685</td>
</tr>
</tbody>
</table>

Storage of the thermometer in ice or in a deep freeze prevented the necessity of recalibration each time the apparatus was used.

The reproducibility of the method is shown by seven readings that were taken on samples of 0.35 Molar sodium chloride. The mean actual depression of freezing point of these seven samples was 1.185°C with a standard error of the mean of ±0.003.

Results

The results from a series of experiments, performed in the manner described, are presented in table 8 and fig. 19. In the graph the results obtained by Freeman and Rigler (1957) for the depression of freezing point of the blood of *S. plena* in relation to the external medium are combined with the present results for the depression of freezing point of kidney fluid.
Fig. 18. Calibration line for Beckman thermometer.

Actual $\Delta$ (°C).

Beckman thermometer reading.
<table>
<thead>
<tr>
<th>Number of observations</th>
<th>Mean actual $\Delta^\circ C$</th>
<th>Standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sea water 17</td>
<td>1.895</td>
<td>0.001</td>
</tr>
<tr>
<td>Kidney fluid 7</td>
<td>1.960</td>
<td>0.021</td>
</tr>
<tr>
<td>Sea water 5</td>
<td>1.390</td>
<td>0.005</td>
</tr>
<tr>
<td>Kidney fluid 16</td>
<td>1.418</td>
<td>0.008</td>
</tr>
<tr>
<td>Sea water 6</td>
<td>1.005</td>
<td>0.008</td>
</tr>
<tr>
<td>Kidney fluid 8</td>
<td>1.028</td>
<td>0.047</td>
</tr>
<tr>
<td>Sea water 5</td>
<td>0.830</td>
<td>0.005</td>
</tr>
<tr>
<td>Kidney fluid 7</td>
<td>0.920</td>
<td>0.025</td>
</tr>
<tr>
<td>Sea water 7</td>
<td>0.595</td>
<td>0.010</td>
</tr>
<tr>
<td>Kidney fluid 17</td>
<td>0.595</td>
<td>0.014</td>
</tr>
<tr>
<td>Sea water 9</td>
<td>0.550</td>
<td>0.004</td>
</tr>
<tr>
<td>Kidney fluid 6</td>
<td>0.549</td>
<td>0.010</td>
</tr>
<tr>
<td>Sea water 9</td>
<td>0.505</td>
<td>0.004</td>
</tr>
<tr>
<td>Kidney fluid 4</td>
<td>0.580</td>
<td>0.015</td>
</tr>
<tr>
<td>Sea water 11</td>
<td>0.478</td>
<td>0.002</td>
</tr>
<tr>
<td>Kidney fluid 8</td>
<td>0.503</td>
<td>0.006</td>
</tr>
<tr>
<td>Sea water 11</td>
<td>0.555</td>
<td>0.001</td>
</tr>
<tr>
<td>Kidney fluid 17</td>
<td>0.738</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Notes: (short equilibration time - see text)
Fig. 19. Relation between the depression of freezing-point of the external medium and the blood and kidney fluid of *Scrobicularia plana*.

- kidney fluid, results from present work.
- Blood, results from Freeman and Rigler (1957).
- Kidney fluid, short equilibration time (see text, page 146).
- Theoretical isosmotic line.
Discussion

The changes in the osmotic pressure of the kidney fluid of lamellibranchs in response to changes in the external medium has not previously been examined in any detail. As stated earlier, Freeman and Llewellyn (1958) demonstrated that at 100% and at 70% sea water the kidney fluid and external medium were isosmotic. The results from this present piece of work show that the kidney fluid is isosmotic with the external medium down to the lowest salinity examined ($\Delta 0.478^\circ$). This figure is approximately equivalent to 25% sea water and appears to be lowest salinity tolerated by S. plana, under laboratory conditions at 15°C, for in excess of two weeks.

As mentioned previously, and as can be seen by reference to fig. 19, Freeman and Rigler (1957) demonstrated that at external medium $\Delta$ of 0.592°C, the blood $\Delta$ was 0.745°C, suggesting that at low salinities S. plana is able to maintain itself hyperosmotic to the medium.

The method used by Freeman and Rigler (1957) to determine the depression of freezing point of the samples was similar to that of Jones (1941) and Gross (1954), and consisted of observing the time taken for complete disappearance of the crystals of frozen samples of
blood and sodium chloride standards. The depression of freezing point of the blood samples was calculated from the curve relating melting time to the freezing point of the standards.

The present method allows for more accurate temperature control of the alcohol bath and for easier observation of disappearance of the last crystal, and is to be preferred for the accurate estimation of the depression of freezing point.

The present results lend support to the doubt of Robertson (1964) as to whether the animals in Freeman and Rigler's (1957) work were showing active control at low external medium concentrations or whether they had not yet come into a steady state with respect to the external medium. The difference between the reading of Freeman and Rigler (1957) for $\Delta$ of blood at an external medium $\Delta$ of 0.592°C and the isosmotic line was 0.153°C, a difference that could be explained by insufficient time for equilibration, although, as previously discussed, the authors had investigated this point in detail. However, a very similar result (external medium $\Delta$ 0.555°C and kidney fluid $\Delta$ 0.738°C) was obtained during the present work for kidney fluid from animals that had been left to equilibrate for a period, similar in length, to that used by Freeman and Rigler (1957). Later experiments,
increasing the time for equilibration to a period in excess of seven
days for animals at low salinities produced the results shown in
table 8 and fig. 19. Thus although the 110 hours equilibration time
that Freeman and Rigler (1957) allowed for animals in 30% and 50% sea
water would seem sufficient with respect to their information on the
equilibration of the animal, it does appear possible that in particular
instances, possibly due to excessive disturbances, this may not be the
case.

With this one exception, the results expressed in fig. 19
demonstrate that the $\Delta$ of blood and of kidney fluid of $S.$ plana are in
close agreement over most of the range, and that the kidney fluid
is isosmotic with the external medium over the complete range
examined. Thus $S.$ plana appears to behave in a similar manner to
estuarine lamellibranchs that have been investigated, by showing little
or no ability to regulate its osmotic pressure other than by closing
its valves and thereby isolating itself from the external medium for
short periods.

It is clear from these results that the environment of

$P.$ subtruncis may undergo a wide variation in osmotic pressure in response
to changes in the concentration of the external medium, sufficient at
length to affect the host. With this fact in mind, the ability of *Proctosces* to tolerate or to regulate to possible variations in osmotic pressure was investigated.
CHAPTER VII

OSMOTIC RESPONSES OF ADULT PROTOZOANS SUBJECTS TO

CHANGES IN THE OSMOTIC PRESSURE OF THE EXTERNAL MEDIUM

Introduction

Little work has been carried out upon the osmotic responses of
trematodes, although they may be exposed to widely different salt
concentrations at different stages of their life history. Digenean
trematodes passing from marine invertebrates to vertebrates are good
examples of this point.

Endoparasitic digeneans are not protected from variations
the osmotic pressure may vary considerably in such habitats as the
alimentary canal and the urinary bladder. In others, for example, the
blood or tissues, it may remain quite constant. With the exception of a
few scattered studies there is little information available on this
aspect of trematode physiology.

Herses (1922) observed that the excretory bladder of an undetermined
cercaria pulsed about twice as fast in freshwater than in Ringer's
solution and that in freshwater the bladder was definitely enlarged.
Junkard and Shaw (1931) demonstrated that certain cercaria from
marine snails showed a marked resistance to hypotonic surroundings,
remaining active in 50% sea water about as long as in 100% sea water.
and also being able to survive for 1 - 4 hours in dilutions as low as 1 sea water.

Information for adult trematodes is also scarce. Schopfer (1932) determined the depression of freezing point of a tissue extract of 

_**P. hepatica**_ and suggested that it was 45% higher than the host's bile. Stephenson (1945 and 1947) found that the survival of the same trematode in vitro was not greatly affected by changes in salt concentration of 58 - 230 mM NaCl. Similarly, Rueding (1950) found that glycolysis and activity of _**S. mansoni**_ was not affected by changes of concentration of NaCl from 68 - 680 mM.

Only recently have weight change studies been made. Goil (1966) worked on _**G. orumenifer**_ in different NaCl solutions ranging from 68 - 136 mM and Knox and Pantelouris (1966) observed the effect on _**P. hepatica**_ of various concentrations of Hedon Fleig medium and Hedon Fleig medium to which different carbohydrates had been added. These later authors found little or no evidence of water regulation but that in vitro _**P. hepatica**_ was able to tolerate changes of external medium from 0.4°C to 0.81°C.

Siddiqi and Lutz (1966) demonstrated that a simple relationship between weight changes of _**P. gigantica**_ and the concentration of the
The primary reason for this experiment was to demonstrate the
range of salinities in which *P. subtenuis* is able to survive. For the purposes of this experiment, the survival of the parasite, free from the host, was investigated. This had the advantage that reactions of the host did not have to be taken into account and definite numbers of Protozoa could be observed. Although this did not provide the normal habitat of the parasite, the close similarity of the kidney fluid and sea water (see page 30) allowed for a relative indication of the true effect.

Materials and method

Parasites were extracted from a sample of *S. plana*, collected from within a small area, and were placed initially into 100% sea water. Once a sufficient number of Protozoa had been obtained, groups of twenty parasites were placed directly into the experimental dilutions. As Weil and Pantin (1931) noted, when working on the free living turbellarian, *Gunda ulvae*, gradual change of salinity rather than abrupt change did not appear to assist subsequent survival.

The experimental dilutions were prepared by the dilution of pasteurised stock sea water (salinity = 34.0%o) with glass distilled water. The accuracy of these dilutions were checked by analysis of the chloride content using a Buchler Cotlove, direct reading,
Chloridometer. The maximum error of dilution accepted was 2.0%.

Although pasteurised sea water was used, the addition of the Protozoa was invariably accompanied by the addition of ciliates and other microorganisms which increased in number rapidly. For this reason all sea water dilutions were changed periodically.

Crystalising dishes containing 100 ml of solution and the sample of Protozoa were covered by a layer of Parafilm and placed in a constant temperature room set to the desired temperature. Investigation of the samples at intervals ascertained the numbers of Protozoa surviving.

Because of the great mobility of the trematodes this was easily accomplished, doubtful cases were tested by gentle prodding with a fine glass rod. Further indication as to whether a Protozoa was dead or alive was provided by the rapid loss of colour at death, the trematodes then assuming an opaque white colour.

Results

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>100%</th>
<th>75%</th>
<th>50%</th>
<th>30%</th>
<th>20%</th>
</tr>
</thead>
<tbody>
<tr>
<td>15±1</td>
<td>54</td>
<td>79</td>
<td>90</td>
<td>82</td>
<td></td>
</tr>
<tr>
<td>10±1</td>
<td>157</td>
<td>160</td>
<td>167</td>
<td>170</td>
<td>95</td>
</tr>
<tr>
<td>5±1</td>
<td>180</td>
<td>290</td>
<td>310</td>
<td>330</td>
<td></td>
</tr>
</tbody>
</table>
Fig 20. Survival of adult Proctoeceps in various dilutions of sea water at 6°C ± 1°C.

Key to figs 20 and 21.
- ○ • survival in 100 %
- ○ ○ survival in 75 %
- ▲ ▲ survival in 50 %
- ▲ ▲ ▲ survival in 30 %
- ■ ■ survival in 20 %
Fig. 21. Survival of adult Proctoeces in various dilutions of sea water at 10°C ± 1°C and 15°C ± 1°C.
Discussion

It is clear from the results (table 9 and figs. 20 & 21) that adult Prootoeoes subtenuis are able to survive for prolonged periods in sea water dilutions ranging from 20% to 100% sea water.

It was observed that in 20% sea water the osmotic pressure increase was sufficient to cause the cirrus to be extruded in a number of individuals. Although this is undoubtedly approaching the lower limit of salinity tolerance, 50% of the trematodes survived for 95 hours and the last one died after 172 hours. Survival was greatest in all dilutions at 6°C ± 1°C, with the last Prootoeoes dying in the 30% and 50% dilutions after 656 hours (28 days).

2. Salinity for optimum survival

As a consequence of carrying out the above experiments to determine the salinity tolerance of Prootoeoes, it was observed that the most prolonged survival occurred in the 30% and 50% sea water dilutions (see table 9 and figs. 20 & 21). This was surprising in respect of the data on the salinities prevailing at Chalkwell, that were presented by Freeman and Rigler (1957). They stated that the variation in salinity during that part of the tidal cycle to which any animal living near mid-tide level would be subjected, was not very great. However, they did suggest that seasonal fluctuations could be
fairly extensive. To illustrate this fact they quoted figures obtained from the Water Pollution Research Laboratory that showed that the salinity at Chalkwell could vary from 25.2%, in a wet winter, to 32.4% in a dry summer.

The present author collected samples of sea water from mid-tide level at Chalkwell (site 5) at periods during 1969, to determine the extent of possible salinity variation at this locality. Samples were collected approximately fifteen minutes after the area was covered by the incoming tide and were subsequently analysed for chloride content by the use of a Buchler Cottlove, direct reading, Chloridometer. The chlorinity values once calculated were converted to give a salinity value by application of the formula (from Harvey 1955).

\[
\text{Salinity (}) \times \text{) } = 0.03 + 1.6050 \times \text{(chlorinity)}
\]

The salinities recorded during 1969 varied between 25.66% and 32.33% and thus closely resembled the figures quoted by Freeman and Rigler (1957). It is thus suggested that these figures are typical of the range of salinities that occurs at Chalkwell and at other collecting sites in the Estuary proper.

The lowest salinity recorded at Chalkwell would be equivalent to a sea water dilution of approximately 75% (when 100% = salinity of 34.09%).
It would therefore appear extremely unlikely that Prootoeoes as a parasite of S. Planus in the Thames Estuary would ever experience the low salinities at which it appears to survive best under experimental conditions (see page 159).

The significance of the increased survival of Prootoeoes at 30% and 50% sea water is therefore obscure. One suggestion for this apparent preference for lowered salinity concerns the 'normal' host of Prootoeoes. If this trematode is primarily a parasite of the hind gut of sparids and labridae, it is interesting to theorise upon the likely osmotic conditions in the gut.

Teleost fish in sea water maintain themselves hyposmotic to the medium and so a parasite of the hind gut would not experience 100% sea water. As shown for Lophius, Anguilla and Myxocephalus (Smith 1930 and 1932) the osmotic concentration decreases down the gut due to relatively more salt than water being absorbed.

Table compiled from Smith (1930)

<table>
<thead>
<tr>
<th>Species</th>
<th>Sea water Δ°C</th>
<th>posterior intestinal fluid Δ°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lophius piscatorius</td>
<td>1.33</td>
<td>0.83</td>
</tr>
<tr>
<td>Anguilla rostrata</td>
<td>1.81</td>
<td>0.75</td>
</tr>
<tr>
<td>Myxocephalus octodecem-</td>
<td>1.85</td>
<td>0.75</td>
</tr>
<tr>
<td>spinosus</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Thus a parasite of the hind gut of Lophius would be living within
an environment with an osmotic pressure approximately equivalent to 62% sea water; of Anguilla, 41% and Myoxocephalus, 40% sea water.

This general picture is also true for the flounder, Platichthys flesus, in which the osmotic pressure of the posterior intestine has been shown to vary approximately between 29% - 45% sea water (Mackenzie and Gibson, 1970). Similarly, the osmotic pressure of the posterior intestine of the cod, Gadus morhua, was shown by Williams, McVicar and Ralph (1970) to be approximately equal to 32 - 63% sea water.

It is impossible to state, without evidence of the osmotic conditions in the hind gut of sparids and labrids, that 30 - 60% sea water closely resembles the normal environment of Protoeceus, but it is very probable that, as with all marine teleosts so far investigated, the osmotic pressure of the hind gut would be substantially less than that of 100% sea water.

There is obviously a danger in drawing too many inferences from experiments on the survival of a parasite in a medium that, however similar to the normal environment, may be substantially different. It is clearly also open to discussion whether P. subtenuis, parasitic within S. plana in the Thames Estuary, could be demonstrating the remains of adaptation to its 'original' host.
Proctoeces, as a parasite of S. plana, can obviously survive at sea water concentrations continually in excess of 75% as is evident from the fact that all the Proctoeces occurring in S. plana at sites 4, 5, 6, 7 and 8 (i.e. in the Estuary proper) are never exposed to lower concentrations. The observation that the survival of adult Proctoeces appears to be increased at lower salinities is thus thought to be somewhat irrelevant to the parasite of S. plana in the Thames Estuary, although it is possible that it represents the remains of an adaptation to a previous environment.

3. Osmotic responses of Proctoeces subtenuis

No information is available as to the osmotic pressure of adult trematodes in different salinity external media. The availability of large numbers of P. subtenuis presented the opportunity to study if any degree of regulation existed in response to the widely different salinities that it had been shown to be able to survive.

Materials and method

The osmotic responses were measured using the depression of freezing point technique described previously. The problem was investigated from two aspects.

1. The depression of freezing point of Proctoeces, free from the
host and equilibrated with external media of different salinities, was ascertained.

2. The Δ of Protooeceae removed from the kidney of S. plana (that had been allowed to equilibrate with different concentrations of external media) was determined. In this case the Δ of the Protooeceae is compared with the Δ of the kidney fluid.

In the first series of experiments the Protooeceae were placed in various dilutions of sea water in 100 ml crystallising dishes and were left at 15°C ± 1°C, for up to 36 hours.

In the second series of experiments, S. plana were equilibrated at 15°C ± 1°C with various dilutions for periods, the length of which was governed by the dilution (see page 136).

In both series of experiments dilutions of approximately 100, 75, 50 and 30% sea water were prepared by the dilution of the stock sea water (salinity 34.09%) with glass distilled water. It was not necessary to test the accuracy of these dilutions initially, as in the case of the survival experiments, because the ultimate estimation of the depression of freezing point of the solution bathing the Protooeceae, whether it was sea water or kidney fluid, provided the information needed.

In the second series of experiments, after the period of
equilibration each S. plana was opened and a sample of kidney fluid taken
and placed in capillary tubes for subsequent analysis, as described
previously (see page 138). This was rapidly followed by the removal of
the Proctoeca, from which any excess external medium was removed by
gentle blotting.

Preparation of the sample

In both series of experiments, once blotted, the Proctoeca were
placed under liquid paraffin on a cavity slide. The next part of the
operation was carried out under a binocular microscope and consisted
of preparing a sample of the fluid for analysis by the method of Ramsey
and Brown (1955).

Obtaining a sample from such a small trematode presented
technical difficulties. It was found most convenient to open the
Proctoeca by the use of a very fine (approximately 100 μ) glass rod
and then to allow the small amount of fluid contents to flow out.
However, it should be kept in mind that these animals are devoid of
any coelomic cavity and thus of free body fluids. Therefore any fluid
obtained was usually contaminated with cellular material. Coating of
the slide with a smear of silicone grease prevented the fluid from
becoming a thin film.
This fluid was then sucked up into a fine capillary tube as described previously (page 138). The samples prepared in this experiment were of the order of $10^{-3} \text{mm}^3$. Once sealed into the fine capillary and protected by a carrier tube the samples were rapidly frozen by solid CO$_2$ and stored in a deep freeze until used for analysis. As discussed by Read and Simmons (1963) this rapid freezing of samples is important as it prevents the increase in osmotic pressure that has been observed to occur when samples have not been so treated.

All samples were obtained with some difficulty, especially those from Proctoeces equilibrated with 100% sea water.

The freezing point depression of the samples was determined using the modified apparatus of Ramsey and Brown (1955) as described previously (see page 138).
### Table 10: Series 1 Experiments

<table>
<thead>
<tr>
<th></th>
<th>Number of Observations</th>
<th>Mean Actual $\Delta \text{°C}$</th>
<th>Standard Error of the Mean</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sea water</strong></td>
<td>5</td>
<td>0.610</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>Proctoeaes</strong></td>
<td>4</td>
<td>0.690</td>
<td>0.017</td>
</tr>
<tr>
<td><strong>Sea water</strong></td>
<td>6</td>
<td>0.590</td>
<td>0.005</td>
</tr>
<tr>
<td><strong>Proctoeaes</strong></td>
<td>4</td>
<td>0.695</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Sea water</strong></td>
<td>4</td>
<td>1.002</td>
<td>0.009</td>
</tr>
<tr>
<td><strong>Proctoeaes</strong></td>
<td>3</td>
<td>1.237</td>
<td>0.016</td>
</tr>
<tr>
<td><strong>Sea water</strong></td>
<td>8</td>
<td>1.435</td>
<td>0.015</td>
</tr>
<tr>
<td><strong>Proctoeaes</strong></td>
<td>2</td>
<td>1.627</td>
<td>0.012</td>
</tr>
<tr>
<td><strong>Sea water</strong></td>
<td>7</td>
<td>1.420</td>
<td>0.095</td>
</tr>
<tr>
<td><strong>Proctoeaes</strong></td>
<td>6</td>
<td>1.584</td>
<td>0.018</td>
</tr>
<tr>
<td></td>
<td>Number of observations</td>
<td>mean actual $\Delta^\circ C$</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>-----------------------</td>
<td>------------------------</td>
<td>-----------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>Kidney fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of <em>S. plana</em></td>
<td>4</td>
<td>0.585</td>
<td>0.017</td>
</tr>
<tr>
<td>Proctoeces</td>
<td>7</td>
<td>0.760</td>
<td>0.019</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of <em>S. plana</em></td>
<td>17</td>
<td>0.740</td>
<td>0.013</td>
</tr>
<tr>
<td>Proctoeces</td>
<td>2</td>
<td>0.725</td>
<td>0.039</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of <em>S. plana</em></td>
<td>11</td>
<td>1.135</td>
<td>0.094</td>
</tr>
<tr>
<td>Proctoeces</td>
<td>3</td>
<td>1.188</td>
<td>0.020</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of <em>S. plana</em></td>
<td>16</td>
<td>1.423</td>
<td>0.008</td>
</tr>
<tr>
<td>Proctoeces</td>
<td>5</td>
<td>1.690</td>
<td>0.048</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Kidney fluid</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>of <em>S. plana</em></td>
<td>12</td>
<td>2.020</td>
<td>0.054</td>
</tr>
<tr>
<td>Proctoeces</td>
<td>1</td>
<td>2.215</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig. 22. Depression of freezing-point of fluid extracted from Proctoece subtenuis in relation to that of the bathing medium (sea water in series 1 experiments and kidney fluid in series 2 expts.).

95% confidence limits plotted for calculated regression line of the combined results.
Results

The line connecting the points in fig. 22 was calculated using all the results: those obtained for the Protococes kept in sea water, free from the host, and those for animals removed from the host just prior to examination. This was done because of the similarity of the two regression lines calculated for the individual sets of data, both lines being very significant for the data (\( P < 0.01 \) - statistical test d, page 31). To illustrate this point values for the \( \Delta \) of Protococes for given values of the \( \Delta \) of the medium (sea water in series 1 experiments and S. plana kidney fluid in series 2 experiments) are compared in the table below.

<table>
<thead>
<tr>
<th>( \Delta ) of bathing medium (°C)</th>
<th>Series 1 Expts. Protococes ( \Delta ) from sea water (°C)</th>
<th>95% ( \Delta ) Protococes conf limits</th>
<th>Series 2 Expts. Protococes ( \Delta ) from host (°C)</th>
<th>95% ( \Delta ) Protococes conf limits</th>
<th>Combined conf limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5</td>
<td>0.598</td>
<td>0.115</td>
<td>0.615</td>
<td>0.271</td>
<td>0.607</td>
</tr>
<tr>
<td>1.0</td>
<td>1.146</td>
<td>0.110</td>
<td>1.153</td>
<td>0.261</td>
<td>1.150</td>
</tr>
<tr>
<td>1.5</td>
<td>1.695</td>
<td>0.115</td>
<td>1.692</td>
<td>0.270</td>
<td>1.693</td>
</tr>
<tr>
<td>2.0</td>
<td>2.243</td>
<td>0.128</td>
<td>2.230</td>
<td>0.294</td>
<td>2.236</td>
</tr>
</tbody>
</table>

Regression line slope (b)  
1.0966

1.0765

1.0855

The difference in the slope of the two regression lines has been shown to be not significant (see statistical test f, page 32). Because of this and on the basis of the similarity shown in the above table and
the overlap of the lines by the calculated 95% confidence limits it was considered that the calculation of the regression line for the combined data was justified. The 95% confidence limits calculated for this line overlap the theoretical isosmotic line (see fig. 22) and thus suggests that at this level of significance the difference between the calculated line and the isosmotic line (0.1°C at an external Δ of 0.5°C and 0.2°C at external Δ of 2.0°C) is insignificant. To determine experimentally the cause of such a difference in such small animals would be extremely difficult and could be due to any one or to a combination of many small molecules. Autolysis can also lead to changes in the osmotic pressure of samples.

The close similarity of the two sets of results from the two series of experiments also suggests that there is no osmotic difference between Protozoa equilibrated in sea water for a period and those equilibrated within the host. This could be considered as evidence that the time allowed for animals, free from the host, to equilibrate in sea water was not excessive in duration; however it is probable that the Δ of a moribund animal or even part of an animal would be the same as the bathing medium. For this reason care was taken to use only those Protozoa which were active and were unimaged.
Discussion

Although the divergence of the calculated line from the theoretical isoosmotic line has been shown to be statistically insignificant, it is interesting to note that a permanent hyperosmotic state has been suggested for a euryhaline ciliate, *Niamienis avidus* that is a facultative parasite of sea horses (Kaneshiro et al, 1969 a & b).

The authors suggest on the basis of the relationship between the rate of fluid output of the contractile vacuole and the osmolarity of the medium that this organelle may have an osmoregulatory function. The fact that the contractile vacuole of the animal was seen to function even in 200% sea water was taken as a suggestion that the cells are hyper-osmotic to the medium. The author states that preliminary depression of freezing point determinations upon intact cells have confirmed this.

Kaneshiro and his fellow workers' explanation of this observation is that it may be a mechanism to obtain water to operate the contractile vacuole and that this organelle may be responsible for the elimination of some metabolic wastes.

The physiology of excretion in trematodes appears to have been almost entirely neglected although it has been stated (Smyth, 1966; Siddiqi and Lutz, 1966) that the excretory system plays a part in osmotic pressure regulation.
Proctoeoses possesses a well developed excretory system but there can only be conjecture as to whether water obtained through the worm being in a permanent hyperosmotic state would be of any value in assisting the elimination of waste metabolites.

In summary it can be stated that Proctoeoses, similar to the majority of marine invertebrates, appears to be isosmotic (to within a Δ of 0.2°C) with its environment. In the event of a change in the concentration of that environment Proctoeoses acts as an osmoconformer, gaining or losing water and salts according to the concentration of the medium. The tolerance of Proctoeoses to wide salinity changes and the inability to regulate to any significant degree, is a measure of the ability of the cellular metabolic processes of the worm to function under widely different conditions.
CHAPTER VIII

OXYGEN TENSIONS IN THE KIDNEY OF SCROBICULARIA PLANA

Consideration of any aspect of the respiratory physiology of a parasite, or any other animal, must be discussed in relation to the oxygen tensions occurring in the environment.

Most of the results of work on the availability of oxygen in parasitic environments has been summarised by von Brand (1946, 1952 and 1966). This work mainly considers the conditions in vertebrate host tissues. The results show much variability and, as has been suggested by Smith (1969), many of the assertions may prove unjustified.

Recent work using oxygen electrodes has provided reliable data. Possibly the best such study to date is that of Crompton et al (1965). These authors demonstrated that the oxygen tensions in the intestine of domestic ducks varied from 25 mm Hg. close to the villi, to less than 0.5 mm Hg. in the centre of the lumen.

The oxygen tension within inter-tidal lamellibranchs has only been investigated in a few species. Von Brand’s assertion (1946) that most intertidal lamellibranchs shut their valves tightly at low tide has been shown to be untrue in some instances.

The other tellinid lamellibranch commonly found on muddy shores,
Macoma balthica, has been shown by Brafield (1963) to move extensively at low water. This author has shown that these movements can be induced experimentally by low oxygen tensions in the substrate, the animal then fully exposing itself on the surface of the sand, when the entire mantle edge is brought into contact with the well oxygenated surface water lying between the ripple marks.

Beyden (1970) has shown that Cerastoderma edule also does not shut its valves tightly at low tide but that it is able to take an air bubble into its mantle cavity and utilise the oxygen in order to continue to respire aerobically. This is in comparison to the behaviour of the closely related C. glaucum which does shut its valves tightly when exposed.

There is some evidence that, to a varying degree, bivalves can respire anaerobically when the tide uncovers them. Obviously facultative anaerobiosis has survival value to intertidal molluscs. Moore (1931) found that Syndosmya (= Abra) alba could survive in deoxygenated water, with which it retained contact with its siphons, for 3½ days. Thamrup (1935) showed that when a number of molluscs were sealed into a tube, none of the Macoma and only a few Mytilus edulis died after a week compared with all the Cardium (= Cerastoderma) and Mya.
Evidence that *Mya arenaria* may build up an oxygen debt when exposed is provided by Van Dam (1935). This author states that normally *Mya* does not remove more than between 5 - 10% of the oxygen from the water passing through its mantle cavity, but after being uncovered by the tide it gradually builds up an oxygen debt that is repaid on re-immersion by increased utilisation of over 20%.

The only recordings of oxygen tensions inside a lamellibranch are provided by Lent (1968). He investigated the mantle cavity fluid oxygen tensions of *Modiolus demissus* during a simulated tidal cycle using a Beckman model 777 laboratory oxygen analyser which was inserted through a 15 mm hole bored in the shell and mantle.

This author showed that this species gapes when exposed at low tide and that it respires aerically but at a lowered rate. The partial pressure of oxygen in the mantle cavity fluid upon exposure was low: 10 - 18 mm Hg., but rose upon inundation to about 105 mm Hg. This latter figure was obtained when the partial pressure of the external water varied between 120 and 145 mm Hg.

To define the extremes of oxygen tension present in the environment of *Prootoeoes* the use of a large electrode of the type used by Lent (1968) and the measurement of the oxygen tension in the mantle
cavity fluid was not sufficient. Unlike all previous investigations, the oxygen tension within the tissue of a lamellibranch was to be investigated. In this case it was to be within the kidney of Scrubiculairia plana.

Materials and method

It was found to be convenient to use the miniature oxygen electrode used by Newell and Courtney (1965); a design similar to that described by Naylor and Evans (1960).

a. Description of the electrode (see fig. 23).

The electrodes constructed by the author consisted of an insulated platinum wire of 0.2 mm diameter sealed with Araldite into silver tubing of 0.3 mm internal diameter and 0.5 mm external diameter. The insulated platinum wire formed the cathode and the silver tube the anode of the electrode assembly. It was found to be convenient to set this electrode into a glass capillary tube to prevent any bending, which might have upset the calibration.

The tip of the electrode was ground flat and polished with fine emery powder. Inspection of the electrode tip under a binocular microscope ascertained when this had been completed successfully. The polished tip was then dipped into a 7% solution of polystyrene in
Fig. 23. Design of the microelectrode.

- Coaxial cable
- Glass holder
- Silver anode
- Insulation
- Platinum cathode
- Polystyrene membrane

0.5 mm.
trichlorethylene, inverted and left to dry, as was recommended by Naylor and Evans (1963). These authors demonstrated that such polystyrene covered electrodes are independent of stirring effects outside the membrane, have a time response of a few seconds and are not poisoned by the tissue.

The small size of these electrodes is the most important consideration when a system is needed for work in tissue. The small size means that there is little disturbance to the tissues and the oxygen consumed by the electrode is small. Courtney and Newell (1965), on the basis of evidence from Kanwisher (1959) calculated that the rate of oxygen consumption for such a microelectrode would be of the order of $1 \times 10^{-4}$ ml. O$_2$/hour if run continuously. With the pulsed system, as used on the present design apparatus, this amount of oxygen would only be consumed after 30 hours use. This rate was considered insignificant with regard to the present investigation.

Once the electrode had been prepared it was used in sea water prior to being used in any investigation. Initially, no current flows between the cathode and anode but, after running, the system becomes conducting and it is then ready for use.

Each electrode prepared in this manner had individual properties
and so each had to be calibrated before use in an electrolyte similar to that in which it was to be used.

The electrodes were used in conjunction with a polarograph. A potential difference of 0.7 volts was applied across the electrode for 0.5 seconds every 15 seconds and the resultant current was amplified and recorded. The pulsing of the applied voltage for only 0.5 seconds extended the life of the electrode greatly by slowing any "plating out". The reading of the resultant current was then held on the meter for 15 seconds, until the next pulse.

The disadvantage with these small electrodes is that the output is very small: of the order of 0.5 µA, which means that any induced currents from such sources as nearby switches are likely to interfere with the reading. On the present design of polarograph the incorporation of an integrator into the circuit helped to overcome this difficulty. Even so, it was found to be convenient to carry out experiments inside a copper box, in a constant temperature room.

b. Properties of the electrode

Because this type of electrode is relatively unused as a tool for biological research, little information exists as to its properties under varying experimental conditions. Naylor and Evans (1960)
had demonstrated a linearity of the calibration line over a range from 0 - 4 atmospheres oxygen partial pressure but for this work it was necessary to test the response of the electrode to oxygen dissolved in sea water. For this reason, before the electrodes could be used, the linearity of the calibration curve had to be established and the effect of temperature on the system ascertained.

**Calibration curve.**

**Method**

250 ml glass bottles, fitted with rubber bungs, had either air or oxygen-free nitrogen bubbled through the sea water that they contained, for various periods, in order to obtain different oxygen tensions. To obtain low oxygen tensions (of the order of 0.1 ml O₂/L) it was found to be necessary to bubble the solutions with oxygen-free nitrogen for periods in excess of two hours.

Once the solution had been bubbled with the appropriate gas for a certain period, the inlet and outlet tubes were sealed and the solution allowed to stand for a time in order that bubbles of gas in the solution would settle and the temperature of the solution would become constant.

The electrode was then sealed, via a hole in the rubber bung
fitted with a 'plug', into each solution in turn and twenty consecutive readings of current output noted.

The complete experiment was carried out in a constant temperature room at $15^\circ C \pm 1^\circ C$ to ensure that no temperature fluctuations had to be taken into account. As a further precaution, the temperature of each solution was recorded by the use of a Coaxark electric thermometer.

After each estimation of oxygen concentration by the use of the electrode, reagents were added to the sample in order to estimate the oxygen present in the solution by the Winkler method. With this procedure, the current output of the electrode could be compared with the known oxygen concentration of the solution.

**Discussion**

From the typical example of a calibration curve expressed in table 12 and fig. 24 it can be seen that a linear relationship exists between current output of the electrode and the oxygen tension in the sea water sample.

This being so it was concluded that for later experiments it was only necessary to use three solutions of sea water with different oxygen tensions to construct a calibration line (for example, see fig 25).
<table>
<thead>
<tr>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
<th>Temperature °C</th>
<th>Oxygen in sample mL/L equivalent pp (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.537</td>
<td>0.0015</td>
<td>14.60</td>
<td>0.106</td>
</tr>
<tr>
<td>0.546</td>
<td>0.0009</td>
<td>14.91</td>
<td>0.143</td>
</tr>
<tr>
<td>0.597</td>
<td>0.0021</td>
<td>15.45</td>
<td>1.67</td>
</tr>
<tr>
<td>0.611</td>
<td>0.0007</td>
<td>14.80</td>
<td>2.07</td>
</tr>
<tr>
<td>0.684</td>
<td>0.0028</td>
<td>15.50</td>
<td>4.13</td>
</tr>
<tr>
<td>0.708</td>
<td>0.0009</td>
<td>16.90</td>
<td>4.50</td>
</tr>
<tr>
<td>0.701</td>
<td>0.0009</td>
<td>14.00</td>
<td>4.79</td>
</tr>
<tr>
<td>0.707</td>
<td>0.0010</td>
<td>16.10</td>
<td>4.85</td>
</tr>
<tr>
<td>0.745</td>
<td>0.0000</td>
<td>16.10</td>
<td>5.90</td>
</tr>
</tbody>
</table>
Fig. 24. Calibration curve of typical microelectrode.

Partial pressure of oxygen (mm Hg).

Polarograph reading.
This was further aided by the observation that although the current output of an electrode for a given oxygen tension may have altered after use, the slope of the calibration line was maintained (see also fig. 25).

Effect on the calibration line of continuous use in tissue

Method

An electrode was calibrated and then inserted through a hole in one valve of a Soro blank, and into the kidney tissue. Once in this position the electrode was left recording for four hours. After this period the electrode was removed and recalibrated (at the same temperature as the initial calibration), using three new sea water solutions containing varying amounts of oxygen.

The two calibration lines so obtained are depicted in table 13 and fig. 25.

Discussion

From the results (table 13 and fig. 25) it was evident that a large change in current output of the electrode for a given oxygen tension was possible after a period of continuous running in tissue. For this reason a different technique was employed in the experiments to determine the oxygen tensions within the kidney of C. plana. This
<table>
<thead>
<tr>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
<th>Temperature °C</th>
<th>Partial pressure of oxygen (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.601</td>
<td>0.0007</td>
<td>15.50</td>
<td>11.50</td>
</tr>
<tr>
<td>0.696</td>
<td>0.0005</td>
<td>15.90</td>
<td>96.40</td>
</tr>
<tr>
<td>0.756</td>
<td>0.0005</td>
<td>16.93</td>
<td>173.70</td>
</tr>
</tbody>
</table>

**Second calibration, after 4 hours continuous running**

<table>
<thead>
<tr>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
<th>Temperature °C</th>
<th>Partial pressure of oxygen (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.380</td>
<td>0.0057</td>
<td>14.80</td>
<td>5.86</td>
</tr>
<tr>
<td>0.430</td>
<td>0.0054</td>
<td>14.90</td>
<td>58.34</td>
</tr>
<tr>
<td>0.537</td>
<td>0.0057</td>
<td>15.30</td>
<td>173.25</td>
</tr>
</tbody>
</table>
Fig. 25. Change in calibration curve after four hours continuous running in tissue.
consisted of only switching on the polarograph and taking readings at intervals (usually 30 minutes). After each interval, 20 consecutive readings were taken, with current flow lasting only 0.5 seconds at each pulse.

Using this method, no change in calibration was observed after running an electrode in tissue for four hours (fig. 26).

The effect of temperature on the performance of the electrode

Method

Oxygen-free nitrogen was bubbled through sea water in a 250 ml. glass bottle, with an oxygen electrode and temperature probe of a Comark electric thermometer sealed into the rubber bung. Whilst bubbling with the oxygen-free nitrogen continued, the bottle was heated in a water bath maintained at approximately 40°C. After a period, of approximately three hours, when the oxygen tension within the sea water had been reduced to a low level and the solution had been heated to the desired temperature, bubbling was stopped and the inlet and outlet tubes were sealed.

The glass bottle was then placed within the copper box in the cold room maintained at 15°C ± 1°C and the experiment commenced. Twenty polarograph readings, and the average temperature of the sea water in the vicinity of the electrode tip during the period of taking
Fig. 26. Calibration line for electrode II before (●) and after (○) 4 hours discontinuous running in tissue.

Partial pressure of oxygen (mm Hg)
the readings, were noted at intervals as the temperature of the sea water in the glass bottle dropped towards that of the cold room.

At the end of an experiment, when the temperature of the sample was no longer decreasing, the electrode was removed and the oxygen content of the sample estimated by the Winkler method. The electrode and temperature probe were then placed in an oxygenated sample and twenty consecutive readings taken at a recorded temperature. The analysis of the oxygen content of this sample by the Winkler method allowed for a calculation of the effect of temperature in terms of oxygen.

Discussion

It is clear from the results for a typical electrode displayed in table 14 and fig. 27 that the temperature of the solution affects the current output of the electrode. Thus, for a given oxygen tension, an increase in temperature would cause an increase in polarograph reading.

The results relating the change in polarograph reading to oxygen tension for a particular electrode, are illustrated in fig. 28.

It is also important to note (fig. 29) that although the current output of an electrode may have altered after much use, the effect of
### TABLE 14

**THE EFFECT OF TEMPERATURE ON THE PERFORMANCE OF ELECTRODES III**

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
</tr>
</thead>
<tbody>
<tr>
<td>35.64</td>
<td>0.509</td>
<td>0.0027</td>
</tr>
<tr>
<td>33.19</td>
<td>0.492</td>
<td>0.0045</td>
</tr>
<tr>
<td>29.42</td>
<td>0.453</td>
<td>0.0039</td>
</tr>
<tr>
<td>26.70</td>
<td>0.438</td>
<td>0.0041</td>
</tr>
<tr>
<td>24.40</td>
<td>0.405</td>
<td>0.0044</td>
</tr>
<tr>
<td>22.10</td>
<td>0.400</td>
<td>0.0046</td>
</tr>
<tr>
<td>20.20</td>
<td>0.372</td>
<td>0.0044</td>
</tr>
<tr>
<td>17.15</td>
<td>0.360</td>
<td>0.0043</td>
</tr>
<tr>
<td>14.10</td>
<td>0.320</td>
<td>0.0051</td>
</tr>
</tbody>
</table>

The sample of sea water, upon analysis, was found to contain 0.155 ml. oxygen per litre (equivalent to a partial pressure of 4.525 mm Hg).
Fig. 27. The effect of temperature on the performance of an electrode.

Position of line calculated by regression line analysis.
Fig. 28. Effect of temperature on a microelectrode in terms of oxygen.

*••* Calculated regression line (as in fig. 27).
(\(P_{O_2} = 4.525\) mm Hg.)

\(\Delta\) Oxygenated sample. (\(P_{O_2} = 119.13\) mm Hg.)

From the graph it can be seen that a change in temperature of 19°C. produced a change in polarograph reading equivalent to a change in oxygen tension in the sample of 114.605 mm Hg. Thus a 1°C change in temperature would cause a change in polarograph reading equivalent to a 6.02 mm Hg. change in \(P_{O_2}\). Thus the temperature coefficient for this electrode was approximately 5% per 1°C change in temperature.
Fig. 29. Effect of temperature on the performance of electrode II before and after use.

Position of lines calculated by regression line analysis.
temperature on the electrode was unchanged.

Thus although these electrodes are sensitive to temperature change it is possible, because of the linearity and constancy of the effect, to compensate for any changes in temperature or, at least, to define the possible errors involved.

Conclusions

With some of the physical properties of the electrode determined it was concluded that they would be suitable for the present investigation, as long as the foregoing facts were noted.

It must be emphasised that each electrode prepared is an individual, and so the response of each electrode to changes in oxygen and temperature has to be determined prior to use.

Oxygen tensions in the kidney of *Sorobicularia plana*

The use of the miniature electrodes presented a unique opportunity to determine the oxygen tensions within the kidney of *S. plana* and thus to demonstrate the range of oxygen tensions occurring within the environment of Prostoceps.

Materials and method

a. Preparation of the *Sorobicularia*

Two holes, each approximately 0.5 cm in diameter, were bored into
one valve of the animal over the area of the kidney, using a dentist's drill bit. One hole was for the insertion of the electrode tip whilst the other allowed for the insertion of a temperature probe of a Comark electric thermometer. By this probe, temperature fluctuations in the vicinity of the electrode could be measured.

The boring of the two holes did not appear to injure the animal as *S. plana*, so treated, were kept alive for as long as normal specimens.

Once the holes had been bored the animal was placed into an aquarium, with one valve embedded in Plastocine to prevent any movement. The tip of a calibrated electrode and the temperature probe were then inserted through the two holes and held in place by a clamp. A small amount of Vaseline applied around both instruments completed the seal.

The aquarium containing the *S. plana* was then filled with sea water to a level sufficient to cover the animal. Aeration of this water in the aquarium ensured that the animal was always availed of fully oxygenated water. The animal was then left in this state overnight to allow it time to function normally. This state was indicated by the extension of the siphons coupled with pumping action.

The entire experiment was performed within a copper box in a constant temperature room maintained at 15°C ± 1°C. This was the same temperature at which the animals had been kept, prior to use.
d. **Experimental procedure**

Initial experiments, with the electrode running continuously, proved unsatisfactory. As stated previously, this was found to be due to the electrode "plating out". For this reason it was found to be convenient to only take readings of current output from the electrode at intervals (usually 30 minutes).

Electrode and temperature probe readings were taken initially for a period with the animal completely submerged, note being taken whether the animal had its siphons extended. As Chapman and Hewell (1956) had shown that the siphon of *S. plana* was open at its tip during extension, this state provided a good indication that the animal was 'open' to the environment.

After this period of immersion, the volume of the sea water in the aquarium was reduced to a level sufficient to expose the *S. plana*. Continued aeration of the remainder of the sea water ensured that the humidity of the air surrounding the animal would be 100% and thus similar to the conditions in a burrow in the mud. A paper hygrometer was used in all experiments to verify this assumption.

During this period of emersion the *S. plana* withdrew its siphons and assumed an appearance characteristic of freshly collected animals,
the free lobes of the mantle edge being clearly visible outside the valves. With the animal in this exposed condition, electrode and temperature readings were taken at intervals.

After the chosen period of exposure the sea water was carefully replaced and aeration continued. Within a few minutes of this taking place the *S. plana* usually extended its siphones and resumed pumping. Electrode and temperature probe readings were then continued, at intervals, until a steady state appeared to have been reached. The electrode was then removed from the animal and re-calibrated. The results of this calibration were compared with the initial calibration to estimate the extent, if any, of reduction in current output of the electrode due to "plating out".

The final part of the procedure consisted of removing one valve of the animal and inspecting the kidney region, by the use of a binocular microscope, to ensure that the electrode had been inserted into the correct area.

**Results**

<p>| TABLE 15 OXYGEN TENSIONS WITHIN THE KIDNEY OF S. PLANA |
|---------------------------------|------------------|------------------|------------------|------------------|</p>
<table>
<thead>
<tr>
<th>Expt.</th>
<th>mean ( P_2 ) when immersed (mm Hg.)</th>
<th>( P_2 ) after 3 hr emersion (mm Hg.)</th>
<th>( P_2 ) after 4 hr emersion (mm Hg.)</th>
<th>mean ( P_2 ) upon re-immersion (mm Hg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>83</td>
<td>30</td>
<td>-</td>
<td>79</td>
</tr>
<tr>
<td>2</td>
<td>60</td>
<td>16</td>
<td>-</td>
<td>63</td>
</tr>
</tbody>
</table>
The results of five experiments to determine the extremes of oxygen tension occurring within the kidney of _S. plana_ are illustrated in table 15. The mean values of the partial pressure of oxygen in the kidney of the immersed animal and the partial pressure in the kidney after three and four hours emersion are tabulated. The animals in experiments 1 and 2 were only subjected to three hours emersion. The set of mean values of the partial pressure of oxygen in the kidney, as determined from the results of the five experiments, are also tabulated.

From the standard error of the mean of the calibration curve measurements, a polarograph reading equivalent to an oxygen tension of zero really means that the oxygen tension was below 0.97 mm Hg. This figure is thus included after each reference to zero in the text.

In tables 16, 17 and figs 30, 31 the results of experiments 1 and 5

<table>
<thead>
<tr>
<th>Expt.</th>
<th>mean $P_2$ when immersed (mm Hg.)</th>
<th>$P_2$ after 3 hrs emersion (mm Hg.)</th>
<th>$P_2$ after 4 hrs emersion (mm Hg.)</th>
<th>mean $P_2$ upon re-immersion (mm Hg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>57</td>
<td>23</td>
<td>'0'</td>
<td>60</td>
</tr>
<tr>
<td>4</td>
<td>72</td>
<td>40</td>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>5</td>
<td>68</td>
<td>'0'</td>
<td>'0'</td>
<td>82</td>
</tr>
<tr>
<td>mean of above results</td>
<td>67</td>
<td>22</td>
<td>3</td>
<td>71</td>
</tr>
</tbody>
</table>
are expressed in more detail as typical examples of the results
obtained for three and four hour periods of emersion.

The pattern of oxygen tension within the kidney of the five S. plana
examined during simulated tidal cycles was found to be similar: When
the animal was immersed the partial pressure of oxygen within the kidney
was found to vary in an individual animal between about 65 mm and 100 mm Hg.
The result expressed in table 15 demonstrates that the mean P O₂ for the
animals investigated was approximately 67 mm Hg. This was when the
partial pressure of oxygen in the external medium varied from
120 - 136 mm Hg.

Withdrawal of the siphons and discontinuance of pumping by the
animal in experiment 1 can be seen from fig. 30 to cause a reduction in
the partial pressure of oxygen in the kidney of approximately 25 mm Hg.
The recovery appears rapid once the siphons are re-extended.

Removal of the sea water from the aquarium caused the exposed
S. plana to withdraw its siphons and to close its valves. The P O₂
in the kidney then dropped: during a three hour period of exposure
to 'O' = 40 mm Hg., (with a mean in the five animals examined of
22 mm Hg) and to 'O' = 10 mm Hg, (with a mean of 3 mm Hg.) during a
four hour period of exposure.
TABLE 16  
OXYGEN TENSIONS WITHIN THE KIDNEY OF S. PLANUS  
DURING SIMULATED TIDAL CYCLE - THREE HOURS EVERSION

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
<th>partial pressure of oxygen (mm Hg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>00.00</td>
<td>0.560</td>
<td>0.0036</td>
<td>92</td>
</tr>
<tr>
<td>00.15</td>
<td>0.568</td>
<td>0.0032</td>
<td>100</td>
</tr>
<tr>
<td>00.45</td>
<td>0.558</td>
<td>0.0026</td>
<td>90</td>
</tr>
<tr>
<td>01.15</td>
<td>0.530</td>
<td>0.0041</td>
<td>66</td>
</tr>
<tr>
<td>01.45</td>
<td>0.530</td>
<td>0.0051</td>
<td>66</td>
</tr>
<tr>
<td>02.30</td>
<td>0.550</td>
<td>0.0056</td>
<td>86</td>
</tr>
</tbody>
</table>

- water removed

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
<th>partial pressure of oxygen (mm Hg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>02.45</td>
<td>0.535</td>
<td>0.0051</td>
<td>70</td>
</tr>
<tr>
<td>03.15</td>
<td>0.527</td>
<td>0.0034</td>
<td>62</td>
</tr>
<tr>
<td>03.45</td>
<td>0.510</td>
<td>0.0049</td>
<td>46</td>
</tr>
<tr>
<td>04.15</td>
<td>0.500</td>
<td>0.0038</td>
<td>38</td>
</tr>
<tr>
<td>05.15</td>
<td>0.490</td>
<td>0.0056</td>
<td>28</td>
</tr>
</tbody>
</table>

- water replaced

<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>mean polarograph reading</th>
<th>standard error of the mean</th>
<th>partial pressure of oxygen (mm Hg.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>05.30</td>
<td>0.515</td>
<td>0.0068</td>
<td>52</td>
</tr>
<tr>
<td>05.45</td>
<td>0.560</td>
<td>0.0050</td>
<td>92</td>
</tr>
<tr>
<td>06.00</td>
<td>0.546</td>
<td>0.0076</td>
<td>80</td>
</tr>
<tr>
<td>06.15</td>
<td>0.544</td>
<td>0.0061</td>
<td>78</td>
</tr>
</tbody>
</table>
Fig. 30. Oxygen tensions within the kidney of *S. plana* during a period of 3 hours emersion.
<table>
<thead>
<tr>
<th>Time (hrs.)</th>
<th>Mean polarograph reading (mm Hg)</th>
<th>Standard error of the mean (mm Hg)</th>
<th>Partial pressure of oxygen (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>00.00</td>
<td>0.585</td>
<td>0.0065</td>
<td>72</td>
</tr>
<tr>
<td>00.30</td>
<td>0.598</td>
<td>0.0050</td>
<td>85</td>
</tr>
<tr>
<td>01.00</td>
<td>0.550</td>
<td>0.0030</td>
<td>37</td>
</tr>
<tr>
<td>01.30</td>
<td>0.539</td>
<td>0.0040</td>
<td>27</td>
</tr>
<tr>
<td>02.30</td>
<td>0.548</td>
<td>0.0032</td>
<td>35</td>
</tr>
<tr>
<td>03.30</td>
<td>0.518</td>
<td>0.0032</td>
<td>'0'</td>
</tr>
<tr>
<td>04.30</td>
<td>0.516</td>
<td>0.0047</td>
<td>'0'</td>
</tr>
<tr>
<td>05.00</td>
<td>0.535</td>
<td>0.0034</td>
<td>22</td>
</tr>
<tr>
<td>06.00</td>
<td>0.579</td>
<td>0.0057</td>
<td>66</td>
</tr>
<tr>
<td>06.30</td>
<td>0.618</td>
<td>0.0049</td>
<td>105</td>
</tr>
<tr>
<td>07.00</td>
<td>0.594</td>
<td>0.0060</td>
<td>82</td>
</tr>
</tbody>
</table>

**Note:**
- Water removed
- Water replaced
Fig. 31. Oxygen tensions within the kidney of *S. plana* during a period of 4 hours emersion.
Fig. 32. Temperature variation inside Scorihcularis as compared with the variation in the surrounding air during a simulated tidal exposure.

Temperature of air inside S. plane.

Temperature of air outside S. plane.

Temperature (°C).
Upon re-immersion, the animal extended its siphons and resumed pumping activity. The partial pressure of oxygen in the kidney then rose, often to a level in excess of the pre-exposure value, but soon recovered to a level similar to that recorded before immersion: between 60 and 82 mm Hg P O₂ with a mean in the five animals examined of 71 mm Hg.

During the duration of the experiments temperature fluctuations within the animal were never found to exceed 1.2° and were simply a reduced copy of the fluctuations of the environment. The temperature fluctuations recorded during experiment 2 are illustrated in fig. 32 to demonstrate this point. Because the observed variation in temperature within the _S. plana_ was small no account was taken of it in the calculations.

During the period of exposure the relative humidity of the air surrounding the _S. plana_, as measured by a paper hygrometer, was never found to fall below 100%.

**Discussion**

The suggestion of Freeman (1962b) that the oxygen tension within the tubular fluid of the immersed _S. plana_ would be in close agreement with that of the overlying sea water appears to be in disagreement with the results of this present investigation. The tubular fluid in the immersed _S. plana_ was found, from the present work, to be approximately
50\% saturated, with respect to the bathing sea water. This would suggest that more considerable barriers to the diffusion of oxygen exist between the mantle cavity fluid and the kidney than was anticipated by Freeman (1962b).

The oxygen tensions recorded in the siphonal immersed S. plana are also lower than those recorded by Lent (1968) for Modiolus demissus. This author recorded an average P\textsubscript{O\textsubscript{2}} of 105 mm Hg. in the immersed animal but it must be emphasised that he was only measuring the mantle cavity fluid oxygen tension, and not in the tissue of the animal as in the present investigation.

The rapid drop of P\textsubscript{O\textsubscript{2}} as shown by Lent (1968) for M. demissus, when emersed, is surprising in view of the fact that the animal gapes in air and would thus be in contact with oxygen at a higher concentration than when in water. The P\textsubscript{O\textsubscript{2}} of 10 - 18 mm Hg. recorded by Lent (1968) for emersed animals was also for the mantle cavity fluid. Diffusion of oxygen into this fluid from the air may be slow but the uptake of oxygen by other parts of the body may be more rapid. The gills, with the constant action of their cilia, would provide a good surface for gaseous exchange even in the exposed animal, and therefore measurement of the mantle cavity fluid oxygen tension could, conceivably, give a false impression of the conditions in the tissue.
Electrodes of the type used by Lent (1968) usually need a large flow of water over them in order to give reliable results. For a Beckman Fieldlab Oxygen Analyser with a sensor tip width of approximately 13 mm, a system similar to that used by Lent (1968), the makers recommend a minimum flow rate past the sensor tip of 1 foot per second. The amount of water remaining in a gaping Modiolus demissus and the flow of water over the electrode tip generated by the mantle cilia may, on this basis, be hardly sufficient to give reliable readings of oxygen tensions.

In S. plana the drop in $P_2$ that occurs in the kidney tissue upon emersion of the animal may be lessened by a reduction in the metabolic rate of the bivalve and possibly by a progressive involvement of anaerobic metabolism. It is also possible that under the conditions of the experiment there is some diffusion of oxygen through the free edges of the mantle, which remain evident even when the animal is closed. As has been discussed in connection with osmotic responses (page 135) substances are able to diffuse through the free edges of the mantle. As pointed out by Newell (1964a), provided that the latter are permeable to oxygen and the oxygen tension of the tissue just below them is lower than that of air, some uptake of oxygen is inevitable. It is considered that because of the probable large extent of the diffusion barrier afforded by
the free mantle edge and the small surface area of free mantle exposed, relative to the size of the animal, that this uptake of oxygen would be minimal and would hardly affect the results.

From table 17 and fig. 31 it can be observed that zero oxygen tensions (less than 0.97 mm Hg.) were recorded in the kidney of *S. plana* after periods of emersion lasting in excess of three hours. The *S. plana*, living on the shore, mainly occur just below mid-tide level and are usually exposed for at least four hours. Therefore there is every likelihood that the oxygen tension within the animal living in a burrow may also fall to the low levels recorded experimentally.

As soon as re-immersed the $P_2$ in the kidney of the *S. plana* rises rapidly and may even increase to a level in excess of the normal value. It is possible that this may be due, in part, to an increased pumping rate or an increased utilisation of oxygen after a period of anaerobiosis, as was observed for *Mya arenaria* by Van Dam (1935).

Although it had been shown that under the conditions of the experiment the $P_2$ in the kidney of *S. plana* could fall to less than 0.97 mm Hg. during a period of emersion in excess of three hours, it was considered that it was possible that under natural conditions of living in a burrow the animal may have been able to utilise the oxygen
in the surface water or water retained inside the animal. To calculate the feasibility of these suggestions it was necessary to know the respiratory rate of *S. plana* and the volume of the water retained in the mantle cavity upon closure of the animal.

**Volume of the mantle cavity**

**Method**

*S. plana* were left for twelve hours in stock sea water at 15°C ± 1°C. After this period, animals that had had their siphons extended were picked out and placed on a flat surface. They were left in this position for approximately five minutes, until they assumed the appearance of freshly collected *S. plana*. The free edges of the mantle lobes were clearly visible outside the closed valves. They were then gently dried and any external water removed, before being weighed. As soon as the weight had been noted the anterior and posterior adductor muscles were severed and the mantle cavity water drained out of the animal. Once this had been accomplished the animal was re-weighed and the dimensions of one valve noted.

The volume of the mantle cavity of the *S. plana* was then obtained by dividing the difference in the two weights by the specific gravity of sea water (salinity 34%) at 15°C. This value is 1.0265.

The results are expressed in fig. 33.
Fig. 33. Mantle cavity volume of Scrobicularia plana.
Respiration rate of *Scrobicularia plana*

**Method**

Animals were collected from Crowstone (site 6) and were stored at 15°C ± 1°C for 24 hours before the first one was used.

The rate of respiration of whole *S. plana* was measured using a Yellow Springs Instrument Company, Model 53, Oxygen Monitor. This consists basically of an airtight chamber surrounded by a water jacket which was maintained at a constant temperature by the use of a Grant water bath. 60 ml. of aerated pasteurised sea water were placed into the chamber which was stirred by a magnetic stirrer. This was left for fifteen minutes to ensure that the temperature of the introduced sea water was 15°C. On commencement of the experiment the *S. plana* was placed into this water and the oxygen probe sealed in.

In all experiments the *S. plana* rapidly extended its siphons and began pumping normally. From this point in time readings of percent oxygen saturation of the sea water in the chamber were recorded at two minute intervals for thirty minutes.

The amount of oxygen in the 60 ml. of sea water, when the recorder registered 100% saturation, was ascertained by carrying out a Winkler estimation. Using this value, the percentage of oxygen consumed by a *S. plana* in thirty minutes could be converted to give a value in terms
of ml O₂ per hour.

At the end of an experiment, animals were removed from the assembly and their size and wet weight noted. Dry weight estimations were performed by placing the flesh of the animal in an oven, at 100°C, until constant weight.

Results

By this method, the respiration rate, in terms of µl O₂/gram dry wt./hour, of thirteen S. plana was ascertained (table 18 and fig. 34). Because there was a tendency for the S. plana to extend its foot and to attempt to burrow for part of the period whilst in the respiration chamber, these rates must be considered to be somewhat in excess of those that would be obtained had the animal not engaged in this activity.

Discussion

For all subsequent discussions, calculations are based upon the data for a 300 mg/gram (dry weight excluding shell) S. plana. This is an animal with a shell width of approximately 2.5 cms. and is approximately five years old.

An S. plana of this size would consume 213 µl O₂ in one hour and its mantle cavity volume would be approximately 1.4 ml. Assuming that
<table>
<thead>
<tr>
<th>Dry weight (mgms)</th>
<th>Oxygen used in 30 minutes (μl)</th>
<th>Respiration rate (μl/gram/hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47</td>
<td>40.6</td>
<td>1,728</td>
</tr>
<tr>
<td>102</td>
<td>56.5</td>
<td>1,108</td>
</tr>
<tr>
<td>108</td>
<td>59.4</td>
<td>1,102</td>
</tr>
<tr>
<td>110</td>
<td>59.4</td>
<td>1,082</td>
</tr>
<tr>
<td>157</td>
<td>95.7</td>
<td>1,216</td>
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<tr>
<td>220</td>
<td>81.2</td>
<td>736</td>
</tr>
<tr>
<td>243</td>
<td>105.8</td>
<td>872</td>
</tr>
<tr>
<td>254</td>
<td>101.5</td>
<td>799</td>
</tr>
<tr>
<td>269</td>
<td>116.0</td>
<td>862</td>
</tr>
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<td>352</td>
<td>107.3</td>
<td>608</td>
</tr>
<tr>
<td>370</td>
<td>130.5</td>
<td>705</td>
</tr>
<tr>
<td>461</td>
<td>156.6</td>
<td>679</td>
</tr>
<tr>
<td>587</td>
<td>120.0</td>
<td>409</td>
</tr>
</tbody>
</table>
Fig. 34. Respiration rate of Scrobicularia plana at 15°C.

Respiration rate (ml/gm/hour).

Dry weight (mgms)

Position of line calculated by regression line analysis.
the water in the mantle cavity, that was trapped when the animal closed, had a salinity of 34.33% and was saturated with $O_2$ at 15°C, then it would contain

$$5.86 \times \frac{1.4}{1000} = 8.2 \mu l O_2$$

* Amount of oxygen dissolved in sea water of salinity 34.33% at 15°C from Nicol (1967), quoted from Fox (1907)

At the respiration rate quoted at 15°C, this amount of oxygen would last the animal for

$$60 \times \frac{8.2}{213} = 2.3 \text{ minutes}$$

From this calculation it can be seen that the amount of oxygen in the water trapped in the mantle cavity of *S. plana* when the animal is closed would not be sufficient to maintain aerobic respiration of the animal at the same rate as when immersed for any significant period.

Another point of discussion is whether, under natural conditions, the *S. plana* is able to make use of the oxygen in the water that remains when the tide uncovers the mud in which it is burrowed. There would not be much oxygen available to the animal from the interstitial water in the black, sulphide mud in which it burrows. Jones (1955) and Braefield (1964) have shown that the interstitial water in this black layer never contains more than 0.68 ml. $O_2/L$, and usually only contains approximately 0.11 - 0.35 ml. $O_2/L$. Assuming an average oxygen content
of 0.35 ml. O₂/L., a 300 mgm S. plana would have to pump, and utilise all the oxygen from, approximately 610 ml of this water per hour in order to maintain its normal respiratory rate. It is extremely doubtful that it would be able to obtain this amount of interstitial water.

The surface water that remains when the tide recedes may be rich in oxygen. As has been shown by Brasfield (1964), the oxygen content may exceed normal saturation levels due to the action of naviculoid diatoms. Thus, according to Brasfield (1964), surface water may contain up to 10.9 ml. O₂/L.

Similarly to Macoma balthica (Brasfield, 1964) S. plana is found to retain contact with the surface layers of the substrate at low tide. This action, as observed by Hughes (1969), is primarily concerned with feeding but it was considered possible by the present author that S. plana could obtain some benefit by pumping the surface water.

Assuming an average value of oxygen in surface water (from those demonstrated by Brasfield, 1964) of 8.13 ml O₂/L then it would require a 300 mgm S. plana to pump, and completely utilise the oxygen from, 26 ml of such surface water per hour in order to obtain 213 μl O₂ needed to maintain its respiratory rate. Unfortunately, it is very unlikely that it would be able to completely utilise all the dissolved oxygen.
Assuming utilisation of oxygen by *S. plana* to be similar to that of *Mya arenaria* (Van Name, 1935) then it would only remove 5 - 10% of the oxygen from the water. Therefore it would need to pump between 260 and 520 mls of surface water per hour to obtain sufficient oxygen to maintain a normal respiratory rate.

This may be possible in certain areas for short periods immediately after the tide has receded, but it is certain that this figure would represent an impossible amount for the *S. plana* at those sites investigated by the author in the Thames Estuary.

It thus seems very probable that *S. plana* living in a burrow in the sulphide layer of the mud is subjected to periods of lack of oxygen when the area is uncovered by the tide. It is possible that during this period the metabolic rate of the *S. plana* is reduced and that there is a progressive involvement of anaerobic metabolism. Further evidence of this may be the discovery by Thorpe (1970) that the pH of the mantle cavity fluid becomes more acid with increased exposure. This could be due to a build up of lactic acid.

**Summary**

From the results discussed in this chapter it appears that the oxygen tension within the kidney of *S. plana* may drop from the immersed...
level of approximately 70 mm Hg, to less than 0.97 mm Hg during a period of exposure, in excess of three hours. It appears from a consideration of the data of the respiration rate, mantle cavity volume and oxygen content of surface and interstitial waters that this could not be prevented (apart from in exceptional areas where large amounts of surface water may remain at low tide) by behavioural reactions of the S. plana. It seems likely that a progressive involvement of anaerobic metabolism may take place.

It thus appears that Protozoa, as a parasite of S. plana, would be subjected to periods when the oxygen tension of its environment drops to zero.
CHAPTER IX

HAMOGLOBIN IN PROCTOCEDES SUBTENUES

Introduction

Many reviews on the subject of haemoglobin and other blood pigments exist in the literature. Of particular use to the present work were the reviews of Prosser and Brown (1962), Jones (1963), Riggs (1965) and the extensive survey of the literature on the occurrence and properties of the haemoglobins of parasitic animals that was carried out by Lee and Smith (1965).

Haemoglobin has been detected in several groups of parasitic animals but it is not necessarily present in all members of a group. As Lee and Smith (1965) pointed out, there is a surprising lack of data on the haemoglobins of trematodes. This must be due in part to the difficulty in obtaining pure samples of a pigment that is often in a low concentration, from an animal that in many cases is small and lacks any body fluid.

Wharton (1941) examined, spectrosopically, the oxyhaemoglobin from two intestinal trematodes of turtles from America: Telorchis robustus and Allasacotoma magnum. The haemoglobin from Gastrothylax crumenifer, Cotylophoron indicus and Fasciola gigantica was examined.
by Goil (1959, 1961). He determined the absorption maxima of the oxy- and deoxyhaemoglobins of these parasites but his values for the haemoglobin of *F. gigantica* are different to those obtained for the pigment extracted from the same parasite by Lutz and Siddiqi (1967). These latter authors also demonstrated that the spectral properties of the parasite's haemoglobin were different from those of the host.

Van Gembergen (1949) extracted the haemoglobin from *Fasciola hepatica* and examined it spectroscopically. He similarly examined the whole *Dicrocoelium lanceolatum* for haemoglobin as did Stephenson (1947b) for *F. hepatica*.

Freeman and Llewellyn (1958) first mentioned the fact that *Proctocephalus subtenus* recovered from *S. plena* was red or pink in colour, and that the colour was generally distributed throughout the tissues of the worm. These authors demonstrated that upon addition of Takayama's fluid to the *Proctocephalus*, the characteristic absorption bands of pyridene haemochromogen were readily observed by the use of a microspectroscope. The band occurred at 558 - 560 nm and the at 525 nm. This formation of a pyridene haemochromogen demonstrated that the coloration of the *Proctocephalus* was due to a native haem pigment. No similar reaction could be detected by these authors for host tissue.
Freeman (1962b) inferred that the pigment was haemoglobin because the pink or red Prootooees turned bright cherry red when exposed to coal gas. Freeman (1963) presented firm evidence that the pigment was haemoglobin. He examined spectroscopically the pigment extracted from ground up Prootooees and showed that the absorbance maxima occurred at 418 nm, 579 nm and 543 nm. These corresponded to the positions of the Soret (\( \delta \)), \( \alpha \) and \( \beta \) band of oxyhaemoglobin. He further showed that the pigment could be deoxygenated by the use of a reducing agent; sodium dithionite. The \( \alpha \) and \( \beta \) bands disappeared leaving a broad region centred on 558 nm.

Thus Freeman (1963) demonstrated that the colouration of the adult Prootooees was due to a pigment which showed the characteristic spectral absorption maxima of oxyhaemoglobin; could be deoxygenated by the addition of sodium dithionite; formed a pyridene haemochromogen and turned cherry red in the presence of coal gas. These findings firmly identified the pigment as haemoglobin.

Freeman and Llewellyn (1958) when originally noting the presence of a haem pigment in Prootooees, could not suggest, on the basis of their information at that time, whether the pigment was involved in the respiratory processes of the parasite. They did mention the interesting
observation of Freeman (1957) that another platyhelminth parasite
of *B. plana*, the turbellarian *Parasorovex acrobiculariae* (Graff)
was also red in colour whereas the closely related species *P. cardii*
that occurs within cockles was not.

Freeman and Llewellyn (1958) further noted that no record of the
occurrence of a pigment had been described by workers who had
recovered *Protozoa* from the hind gut of a fish; an environment
that Freeman (1962) considered might more warrant the possession of
a respiratory pigment.

It is further interesting that no mention of colouration of the
parasite occurs in the descriptions of *Protozoa* recovered from
various invertebrates. This applies to the description of *Protozoa*
from *B. plana* in Germany by Loos-Frank (1969). This is surprising in
view of the fact that it is the most immediately noticeable feature
of *Protozoa* recovered from *B. plana* in the Thames Estuary.

Because the presence of haemoglobin in *Protozoa* recovered from
*B. plana* in the Thames Estuary appeared to be an unusual occurrence
and that it might also apply to another parasite in the same animal,
it was considered by Freeman and Llewellyn (1958) that the investigation
of haemoglobin in *Protozoa* might also demonstrate something of the
metabolic processes of the host. It was for this reason that the present author, as well as investigating the haemoglobin of _Protozoa_ determined the oxygen tension occurring within the kidney of _S. plana_.

**Materials and method**

**a. Extraction of the haemoglobin**

Because of the small size of the adult _Protozoa_ and the small amount of haemoglobin in each worm, it was necessary to secure in the region of 300 to 400 large _Protozoa_ for each haemoglobin experiment. This involved the collection and examination of between 150 and 200 _S. plana_. Once extracted the trematodes were placed in stock sea water and kept overnight at approximately 5°C.

At the commencement of the experiment the worms were removed from the sea water and placed in a glass tissue grinder. They were rapidly washed with distilled water to remove sea water that could possibly cause precipitations with the buffer. The distilled water was then pipetted away and the worms were ground up in a small quantity (approximately 1 ml.) of selected buffer, the tissue grinder being kept in ice to prevent any denaturation of the haemoglobin.

_Sorensen, Na₂HPO₄ + KH₂PO₄_ buffer (Hale 1965) was found to be the most suitable buffer and was consequently used for the majority of
the investigations and the pH was varied in different experiments.

Once the worms were sufficiently ground up the homogenate was transferred to a centrifuge tube. The homogenate was then centrifuged in a M.S.E. 24, High Speed refrigerated centrifuge for 30 minutes at 24,000 r.p.m. (70,000 r.o.f.) at 3°C ± 1°C.

At the end of this period the tube was removed from the centrifuge head and placed in ice. Great difficulty was experienced at this point because the pale pink supernatant fluid was always found to be capped by a very fine, light, white precipitate. The initial technique was to use a fine Pasteur pipette to draw off the clear pink fluid from beneath the precipitate but the slightest vibration of the centrifuge tube would cause the precipitate to become dispersed throughout the otherwise clear, pink, haemoglobin solution.

This precipitate always caused much difficulty and made the early preparations of Proctococc haemoglobin useless. The use of Borate or Tris buffers instead of the usual phosphate did nothing to alter its occurrence or volume. The fact that the total volume of the pale pink supernatant was generally less than 2 mls. meant that many methods of purification were not available for use in trying to rid the supernatant of this precipitate. Most methods of purification would have led to a loss in the haemoglobin content of the solution or to a further dilution.
of the sample, either of which would have proved disastrous.

Initially, one of the most successful methods was found to be to employ a very small sintered glass funnel of about 2 ml capacity. On top of the sintered glass was placed a portion of a 0.22 μ pore diameter, millipore filter. On top of this was placed approximately 1 cm of Celite 545, a diatomaceous filter. This was dampened by the addition of a small amount of the same buffer as was used for the extraction, in order to ensure that little or no haemoglobin solution would be absorbed by the filter.

The supernatant fluid was then slowly ultrafiltrated through this filter and re-collected. Care had to be taken not to reduce the pressure too greatly on the collecting side in order to prevent the haemoglobin solution boiling under reduced pressure and thus becoming denatured.

In certain instances this technique proved to be successful (fig. 35) but the fineness of the precipitate meant that this was in the minority of cases, even when two or three successive attempts of filtration were employed on a single sample. This technique was found to be extremely successful in clearing the solutions of haemoglobin obtained from the blood of many other animals.
Fig. 35. Absorption spectrum of a solution of the oxyhaemoglobin of Proctoeetes before and after the use of Celite 545.
The discovery that this fine white precipitate was mainly fatty in nature led the way for the most successful method of purification. After high speed centrifugation the pale pink supernatant fluid was collected with as little contamination as possible. To this solution was added an approximately equal volume of a 3:1 mixture of Analear Ether and Chloroform. This mixture was then shaken before being centrifuged at about 5,000 r.p.m. in a M.S.E. bench centrifuge for five minutes. At the end of this period the precipitate was generally seen to have disappeared and the result was a cloudy layer of ether/chloroform overlying a clear pale pink fluid. It was not usually necessary to repeat this procedure.

The clear pale pink solution could be easily pipetted from beneath the overlying ether/chloroform mixture. Upon spectroscopic examination, the absorption spectrum of a solution of Prostoeas haemoglobin (fig. 36) and of Tubifex haemoglobin (see page 255), treated in this manner appeared characteristic of a normal oxyhaemoglobin and there was no evidence of denaturation or the formation of ferric haemoglobin derivatives (see fig. 36).

By the use of this method of purification 1 - 2 mls of a clear pink solution was obtained. It was not found to be possible to
Fig. 36: Absorption spectrum of Prostanes oxynemoglobin.

1. After partially successful treatment with Ether / Chloroform.

2. After further treatment with Ether / Chloroform.
concentrate this solution. Attempts were made using Sephadex G-25, as suggested by Flodin et al. (1960) and Deutsch et al. (1963) but the loss of haemoglobin was found to be prohibitive.

The fact that such a small volume of haemoglobin solution was obtained in the initial experiments made it impossible to use standard 1 cm² glass spectrophotometer cuvettes. Cuvettes had to be constructed that would still have an equally long light path length but that would permit the use of smaller total volumes of solution.

b. Special cuvettes to permit the use of small volumes of solution

These are illustrated in fig. 37. The main body of the cuvette was made out of aluminium. The 0.8 mm diameter hole was carefully positioned to ensure that the light beam of the Beckman DB recording spectrophotometer (that was used in all early experiments) would pass through without hindrance when the machine was used on medium slit width.

On the back and on the front of the aluminium block was glued a 1.2X5 cm strip of coverslip. The glue used was Bostik clear adhesive, diluted with acetone. This was applied in an even smear with a paint brush. Care was taken to allow the adhesive to dry well and for any excess acetone to evaporate before the cuvettes were used. This is particularly necessary as acetone can cause the separation of the haem
Fig. 27. Design of cuvette.

KEY:

A - ALUMINIUM CUVEETE.
B - BLACKENED BRASS FRONT PLATE.
C - COVERGLASS.
from the globin (Patel and Spencer, 1963).

Once the strips of coverslip were firmly glued in position the matt black front plate, with a 0.6 mm diameter hole bored in the correct position, was inserted between the front grooves. The front plate was made of brass and was blackened chemically by immersing it in 50 mls of a hot solution containing 2.2 grms. of lead acetate and 2.2 grms. of sodium thiosulphate.

Two cuvettes were prepared in this manner: one to contain the experimental solution and one, exactly similar, to act as the reference cuvette. The solutions were introduced into the chamber of these cuvettes by a fine Pasteur pipette that was inserted down the central groove.

It was found to be convenient for purposes of cleaning to remove the strips of coverslip after each period of use and to replace them with new strips when next needed. To further ensure that the two cuvettes were very similar in optical properties, two strips of coverslip were cut out of each coverslip and one of these strips was used for the specimen cuvette and one for the reference cuvette.

**Performance of the special cuvettes**

These cuvettes were found to give very good results. Care was
taken before every experiment to ensure that the two cuvettes were matched. This was accomplished after comparing cuvettes against each other in a Beckman DB recording spectrophotometer, when both contained only phosphate buffer. The results always showed that there was only a maximum of a 2% difference in the percentage transmission of each cuvette with change in wavelength from 700 to 350 nm.

The exact light path length of these spectrophotometer cuvettes was ascertained by comparing the absorbance of a solution of acridine orange at 492 nm with the absorbance of the same solution in standard 1 cm$^2$ glass cuvettes (see fig. 38). The cuvettes were found to have a light path length of 6.0 mm.

These cuvettes were used in conjunction with a Beckman DB recording spectrophotometer in all early experiments. In the subsequent experiments the more successful extraction technique and the use of a Unicam S.P. 800 ultraviolet spectrophotometer allowed for the use of standard 1 cm$^2$ silica glass spectrophotometer cuvettes.

Wavelength check

Before a series of experiments were begun the accuracy of the wavelength dials of either spectrophotometer was checked using a didymium filter. The position of the peaks of absorbance as recorded were then
Fig. 38. Absorption spectrum of a standard solution of Acridine orange in standard 1 cm$^2$ cuvette (-----) and in constructed cuvette (-----), of unknown light path length.
checked against the positions as quoted in the service manuals. The machine in question was adjusted in the event of a difference in the observed and standard values.

Results

Absorption spectra

The spectrum of the solution obtained by the extraction technique was ascertained. The spectrum (fig. 39 & 40) had all the characteristics expected of a solution of oxyhaemoglobin, with a large peak of absorbance (the Soret or \(\alpha\) peak) in the violet and two lesser, but characteristic peaks in the visible region of the spectrum. These latter peaks are referred to as the \(\alpha\) and \(\beta\) peaks. The exact position of each of these peaks of absorbance is shown in the table and is compared with the values for the oxyhaemoglobin of other digenetic trematodes for which information is available. The exact position of the peaks of absorbance are based upon the results of ten experiments. The position of the peaks represents the mean position calculated from the results of five manual estimations during each experiment using the Beckman DB recording spectrophotometer and from fifty drawn, normal and scale expanded, spectra obtained using both the Beckman DB and the Unicam S.P. 800 spectrophotometers. Many of these drawn spectra were obtained by the
### TABLE 19

**ABSORPTION MAXIMA IN nm OF DIGENEA HEMOGLOBINS.**

<table>
<thead>
<tr>
<th>Species</th>
<th>pH</th>
<th>alpha</th>
<th>beta</th>
<th>Soret</th>
<th>Authority</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Protooeces subtenuis</em></td>
<td>7.2</td>
<td>579</td>
<td>541</td>
<td>417</td>
<td>Present work</td>
</tr>
<tr>
<td></td>
<td>6.04</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Proctoeo. subtenuis</em></td>
<td>8.4</td>
<td>579</td>
<td>543</td>
<td>418</td>
<td>Freeman, 1963</td>
</tr>
<tr>
<td><em>Telorchis robustus</em></td>
<td></td>
<td>575</td>
<td>540</td>
<td></td>
<td>Wharton, 1941</td>
</tr>
<tr>
<td><em>Allassostoma magnum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Fasciola hepatica</em></td>
<td>7.17</td>
<td>580</td>
<td>540</td>
<td>543</td>
<td>Stephenson, 1947</td>
</tr>
<tr>
<td></td>
<td></td>
<td>580 - 1</td>
<td></td>
<td></td>
<td>Van Grembergen, 1949</td>
</tr>
<tr>
<td><em>Gastrothylax orumanifer</em></td>
<td>573.5</td>
<td>539.5</td>
<td></td>
<td></td>
<td>Goil, 1959</td>
</tr>
<tr>
<td><em>Fasciola gigantica</em></td>
<td>586</td>
<td>540</td>
<td></td>
<td></td>
<td>Goil, 1961</td>
</tr>
<tr>
<td><em>Cotylophoron indicum</em></td>
<td>585.2</td>
<td>555.8</td>
<td></td>
<td></td>
<td>Goil, 1961</td>
</tr>
</tbody>
</table>
Fig. 39. Absorption spectrum of oxy- and deoxyhaemoglobin of Proctococcus.
Fig. 40. Absorption spectra - scale expanded (X 5) to show position of peaks.

1. Oxyhaemoglobin.
2. Deoxyhaemoglobin.
superimposing of ten consecutively run spectra. The maximum variation in the position of any peak recorded in any of the experiments was of the order of two nanometers.

Discussion

All the results of the investigations of haemoglobin from trematodes show, not surprisingly, a general similarity in the position of their absorbance peaks.

From the illustration of a spectrophotometer trace of the oxyhaemoglobin of Proctoeces figured in the paper of Freeman (1963) it is suggested that he was unable to purify his sample of haemoglobin to the extent found possible during the present work. This is evident from the flat nature of the alpha and beta peaks and the general increase in optical density towards the shorter wavelengths. Even without taking this into account it is clear that there is good agreement between the two sets of data. There was only 2 nm difference in the position of the beta peaks and a 1 nm difference in the position of the Soret peaks.

One feature of the absorption spectrum of the oxyhaemoglobin of Proctoeces that was evident even in the most pure solutions, was the greater absorbance of the beta peak than the alpha peak. This feature
is unusual but it is in accord with the characteristics of the haemoglobin extracted from Ascaris and Strongylus (Davenport 1949 a & b), root nodule haemoglobin (Keilin and Wang, 1946) and from the trematode Fasciola gigantica (Lutz and Siddiqi 1967). The significance of this property has not been elucidated by research workers, but it is worth noting that the haemoglobins of Strongylus, Ascaris and root nodules are all very resistant to deoxygenation, a characteristic that will also be shown to apply to the haemoglobin of Protozoa.

The absorption spectrum of deoxyhaemoglobin

Deoxygenated haemoglobin was obtained chemically by the use of a reducing agent. Sodium dithionite was added in exactly equal amounts (10 milligrams) to the equal volumes of Protozoa oxyhaemoglobin and to the phosphate buffer in the reference cuvette. This equal addition of sodium dithionite to each solution was to ensure that the strong absorbance peak of sodium dithionite that occurs towards the blue end of the spectrum did not interfere with the absorption spectrum being investigated.

The exact position of the peaks of absorbance are based on the results from ten experiments. This includes five manual estimations during each experiment and a total of twenty normal and scale expanded
drawn spectra. Scale expanded spectra revealed that the deoxygenated peak was composed of two smaller peaks. These were not lost with time or with further additions of ten mgms of sodium dithionite and so are recorded in the following table.

**TABLE 20**

<table>
<thead>
<tr>
<th>species</th>
<th>pH</th>
<th>visible</th>
<th>Soret</th>
<th>Authority</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Protoacces</em></td>
<td>7.2 -</td>
<td>566</td>
<td>550</td>
<td>429</td>
</tr>
<tr>
<td><em>subtenuis</em></td>
<td>8.04</td>
<td></td>
<td></td>
<td>Present work</td>
</tr>
<tr>
<td>Approx. centre</td>
<td></td>
<td></td>
<td></td>
<td>of peak</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>558</td>
<td></td>
</tr>
<tr>
<td><em>Protoacces</em></td>
<td>8.4</td>
<td>558</td>
<td>431</td>
<td>Freeman, 1963</td>
</tr>
<tr>
<td><em>subtenuis</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Telorchis</em></td>
<td>8.4</td>
<td>558</td>
<td>431</td>
<td>Freeman, 1963</td>
</tr>
<tr>
<td><em>robustus</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Allassostoma</em></td>
<td>6.8</td>
<td>545</td>
<td></td>
<td>Wharton, 1941</td>
</tr>
<tr>
<td><em>magnum</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Gastrothylog</em></td>
<td>6.8</td>
<td>560</td>
<td></td>
<td>Goil, 1959</td>
</tr>
<tr>
<td><em>orumenifer</em></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

It is evident from table 20 and fig. 39 that the centre of the peak of absorbance of deoxyhaemoglobin in the visible region as recorded during the present work corresponds well to that recorded by Freeman (1963).

This is despite the fact that it is clear from the spectrophotometer trace figured by this latter author, that he was dealing with a rather
dilute and impure solution of deoxyhaemoglobin. This is evident from
the flat nature of the peak of absorbance in the visible region of the
spectrum that is hardly discernible from the general slope of the
spectrophotometer trace. The overall increase in the optical density
of the solution that occurred after the addition of the sodium dithionite
suggests some contamination, possibly due to precipitation.

When, during the present investigation, the oxyhaemoglobin of
Proteoeeus was deoxygenated with sodium dithionite there was no overall
increase in absorbance and the peak in the visible region was more
discernible. This was especially so with samples obtained later on in
the research programme.

This is important because Benesch et al (1964) state that the prepar-
ation of deoxyhaemoglobin by the use of sodium dithionite can produce
chemical changes in the haemoglobin molecule. They state that
contamination of a sample of deoxyhaemoglobin by methaemoglobin is
evident if there is a shoulder to the spectrum between 410 and 415 nm and
if there is a decrease in absorption at 430 nm. Neither of these two
characteristics were evident in the spectra of Proteoeeus
deoxyhaemoglobin produced by the addition of sodium dithionite and so
the spectra can be assumed to those of deoxyhaemoglobin free from
contamination by methaemoglobin.

Scale expansion of the spectrophotometer trace of the spectrum of the later samples revealed that the single peak in the visible region was composed of two smaller peaks (fig. 40). Experiments were carried out in an attempt to obtain a spectrum lacking these two components. Further additions of 10 milligrams of sodium dithionite to the solution did nothing to alter the spectrum. It was not altered with time or after the addition of a portion of the impure extract of Protozoa haemoglobin.

This double peaked spectrum of deoxygenated haemoglobin is not unique to Protozoa. It has been noted in several other animals. Wittenberg, Briehl and Wittenberg (1965) confirmed its existence in the following annelids:

- Lumbricus 549 563
- Arenicola 552 563
- Nerela 550 565 Wittenberg, Briehl and Wittenberg, 1965
- Tubifex 548 563
- Aphrodite nerve 549 566
- P. subtenuis 550 566 Present work

Wittenberg, Briehl and Wittenberg (1965) obtained this two banded spectrum when the haemoglobin solution from Aphrodite nerve was equilibrated with nitrogen and it was not changed by the addition of sodium dithionite. They showed that the nitrogen equilibrated solution
was fully deoxygenated and free from ferric haemoglobin. They also found
the spectrum was unchanged from pH 5 - 9.3. These authors then
proceeded to confirm reports of similar occurrences in annelids, as
discussed above.

Wittenberg, Wittenberg, Stolsberg and Valenstein (1965) showed that
this unusual spectrum was obtained in muscle slices of _Basycon_ and
_Aplysia_ (from only one natural population) that were placed in anaerobic
conditions. The peaks of absorbance were at 570, 548 and 422 nm and
the reaction was reversed upon admission of air. They referred to this
haemoglobin as the 422 derivative because of the position of the Soret
band. They also showed that this spectrum could be induced from the
oxyhaemoglobin of the muscle slices of _Aplysia_ (from another population),
that would have under anaerobic conditions given a normal deoxygenated spectrum,
by the use of 0.1 M sodium azide or 0.01 M sodium cyanide. This was also
reversible.

Although these observations have been made, the reason for this
double peaked absorption spectrum of deoxyhaemoglobin is unknown. Wittenberg,
Briehl and Wittenberg (1965) did not agree with the claim of Scheler (1960)
that it could be related to the presence of non-haem iron in the molecule
because of the discovery that the iron-haem ratio in electrophoretically
purified _Tubifex_ and _Lumbricous_ haemoglobin was
unity. Wittenberg, Wittenberg, Stolsberg and Valenstein (1965) also ruled out an earlier suggestion (Wittenberg, Brown and Wittenberg 1965) that the 422 derivative of haemoglobin could be a mixture of ferric haemoglobin with other forms.

Again it appears that this unusual spectral characteristic of the deoxygenated haemoglobin of Proctotrupes is found in other animals whose haemoglobin is resistant to deoxygenation. Aphrodite nerve haemoglobin is half saturated with oxygen at a partial pressure of 1.1 mm Hg. (Wittenberg, Briehl and Wittenberg, 1965). The values for the half saturations of the annelid haemoglobins are similarly below 4 mm Hg. (Prosser and Brown, 1962).

Dissociation curve

For any consideration as to the function of a particular haemoglobin, the partial pressures at which it loads and unloads its oxygen must be known. The affinity for oxygen is expressed as the partial pressure at which half the molecules are oxygenated; this is abbreviated to the $P_{50}$ and can be more accurately determined than the pressure of loading or saturation.

Some of the spectral characteristics of the extracted haemoglobin from Proctotrupes have been shown to be consistent with the properties of
haemoglobins known to have a high oxygen affinity. Freeman (1963) also commented that the time taken for the deoxygenation of a sample of Prootoese oxyhaemoglobin by sodium dithionite was much longer than that necessary to deoxygenate a comparable sample of human haemoglobin. He stated that the general impression given was that the oxyhaemoglobin of *Prootoese* was very resistant to deoxygenation.

The present author set out to determine under what conditions, if any, the extracted oxyhaemoglobin from *Prootoese* would unload its oxygen.

**Apparatus**

Because of the small quantity and dilute nature of the solutions of *Prootoese* oxyhaemoglobin that were obtained in the initial experiments, attempts were made to devise a micromethod for the determination of the dissociation curve. The ability with the later more successful extraction procedures to obtain about 2 mls of dilute oxyhaemoglobin solution allowed for the use of a tonometer method similar to that described by Allen, Guthe and Wyman (1950).

**Description of the tonometer (see fig. 41)**

The tonometer was made of silica glass and consisted of a large chamber (A) to which was fused a three way tap for the entry of gases.
Fig. 41. Diagram of Tonometer. (Not to scale.)
One arm of the three way tap was fitted with a Bunsen valve, which made an excellent one way valve system that could be used to allow excess gas in the tonometer to escape.

Fused to the large chamber A was another chamber B. This chamber was where the equilibration of the gases and the haemoglobin solution was carried out. Chamber B was furnished with a side arm.

To chamber B was fused a silica glass 1 cm² spectrophotometer cuvette (C) which could be inserted into the light path of either the Beckman DB recording spectrophotometer or the Unicam S.P. 800 spectrophotometer. The total volume of the tonometers so constructed was in the region of 450 - 500 ml. Special lids were constructed by the author out of thin gauge aluminium sheeting for each of the spectrophotometers. These allowed for the insertion of the tonometer into the sample compartment of either machine. The close fit of the matt black lids and the covering of the tonometer, when inserted, with a black cloth prevented any stray light from entering the sample compartment of the spectrophotometers.

**Experimental technique**

The principle of the technique and the procedure are described by Allen, Guthe and Wyman (1950) and are well illustrated by Riggs (1951).
The basic assumption in the spectrophotometric determination of the degree of oxygenation is that the change in absorbance at any wavelength is linearly related to the fraction of the total number of ferrohaem groups oxygenated.

The experimental procedure was, in the light of experience, somewhat modified from that described by Allen, Guth and Wyman (1950), although the basic procedure was very similar.

The oxyhaemoglobin was introduced through the side arm of chamber B using a Pasteur pipette whilst the tonometer was kept in a horizontal position. The haemoglobin was allowed to run into chambers C and B but was never allowed to enter chamber A. The next step in the procedure was to remove all the air from inside the tonometer and to replace it with oxygen free nitrogen. This was accomplished in one of two ways:

In many experiments the system suggested by Allen, Guth and Wyman (1950) of partially reducing the pressure inside the sealed tonometer followed by the addition of oxygen free nitrogen (100 white spot containing $10^{-3}$ ml $O_2$ per 100 ml of gas) was used. A manometer attached to the water vacuum pump ensured that the pressure reduction was insufficient to cause the haemoglobin to boil under reduced pressure
and thus possibly denature. Pressure reductions of approximately 100 mm Hg. were used in the experiments.

In some experiments an alternative technique was employed. This entailed leaving the solid glass stopper out of the side arm of chamber B. The oxygen free nitrogen supply was connected up to one arm of the three-way tap and the air, in the horizontal tonometer, 'flushed out' by blowing the oxygen free nitrogen straight through. The tonometer was gently rotated whilst in the horizontal position and the slow gas flow continued until complete deoxygenation of the sample. This was tested, in all experiments, by placing the cuvette of the tonometer in a spectrophotometer and by determining the absorption spectrum. If the spectrum so obtained was not identical with the spectrum of a similar sample of haemoglobin deoxygenated by the addition of sodium dithionite then further equilibration with oxygen-free nitrogen was carried out.

When, in the experiments using the second technique, complete deoxygenation was achieved the gas flow was suspended and the side arm of chamber B sealed off.

This latter technique was found to be successful and did not expose the haemoglobin to many pressure changes. Any evaporation of the
haemoglobin that occurred during this period of equilibration (or
during the period of equilibration in the method of Allen, Guthe and
Wyman, 1950) did not matter from the point of view of the subsequent
calculations because the first reading of absorbance was only taken when
deoxygenation was complete and after that time the apparatus was sealed.

When the haemoglobin solution had been completely deoxygenated,
by either method, the pressure inside the tonometer was reduced by an
amount, sufficient to allow the subsequent additions of air. The extent
of the pressure reduction was governed by the properties of the
haemoglobin being investigated. A haemoglobin with a low $P_{50}$ would
only need the addition of a small volume of oxygen to completely saturate
it and therefore only a small reduction in internal pressure would be
necessary to accommodate the introduced gas. Conversely a haemoglobin
with a high $P_{50}$ would need a larger volume of oxygen to bring about
complete reversal to oxyhaemoglobin and therefore a greater reduction
in pressure would be necessary. By ensuring that the air was
introduced into a partial vacuum, no gas mixture had to be expelled
via the Bunsen valve after each addition in order to keep the atmosphere
in the tonometer at atmospheric pressure or to make room for further
additions.
Once the pressure had been reduced in the tonometer (containing only oxygen free nitrogen and deoxyhaemoglobin) it was inserted into the sample compartment of a spectrophotometer. A spectrum of the deoxyhaemoglobin was drawn or the absorbance noted at various wavelengths. In practice, for the purposes of calculations, the absorbance was noted at three wavelengths in the visible region of the spectrum, where the greatest difference in the spectrum of oxyhaemoglobin and deoxyhaemoglobin occur. These were at the positions of the alpha and beta peaks of oxyhaemoglobin and at the peak of deoxyhaemoglobin. Thus the absorbance of the deoxygenated haemoglobin at one of these positions was, for the purposes of the subsequent calculations, that of O₂ saturated haemoglobin.

When the absorbance had been noted the tonometer was removed from the spectrophotometer and, using a hypodermic syringe, a volume of air was injected via the rubber tubing on the side arm of chamber B. The tonometer was then left, gently rotating in a horizontal position for fifteen minutes to allow equilibration to occur. At the end of this period a further reading of absorbance was noted. Leaving the haemoglobin to equilibrate for a further period of fifteen minutes did not alter the reading of the absorbance.
This procedure of introducing known amounts of air and subsequently determining the change in absorbance was continued until no change in absorbance was achieved with two successive introductions of air. The taps of the tonometer were then opened to admit air and to ensure that complete oxygenation had occurred.

Thus at the completion of an experiment a set of absorbance readings or a 'family of curves' were obtained, each one having been recorded after an admission of a known volume of air. Knowledge of the temperature, barometric pressure, the volume of the tonometer and the proportion of oxygen in air allowed for the calculation of the partial pressures at which the various stages of oxygenation had occurred.

Calculation

The percent saturation ($Y$) of the haemoglobin for each absorbance was calculated using the following formula (from Riggs 1951):-

$$\frac{Y}{100} = \frac{K_r - K}{K_r - K_o}$$

where:
- $K_r$ - Absorbance of deoxyhaemoglobin at the selected wavelength
- $K_o$ - Absorbance of oxyhaemoglobin at the selected wavelength
- $K$ - Absorbance of unknown at selected wavelength

The partial pressure of oxygen ($P_{O_2}$) in the tonometer after each injection of air was calculated by the formula (from Riggs, 1951):-
\[
P_{O_2} = \frac{T}{V} \left( \frac{P_o V_o}{T_o} - nR \right)
\]

where:
- \( T \) - temperature in tonometer
- \( T_o \) - room temperature
- \( V \) - volume of tonometer
- \( V_o \) - volume of air injected
- \( n \) - number of molecules oxygen combined with haemoglobin
- \( R \) - gas constant
- \( P_o = 0.2093 (P - H P) \)

where
- \( P \) - Barometric pressure
- \( H \) - Relative humidity
- \( H_p \) - water vapour pressure

**Performance of the apparatus**

Because of the great difficulty experienced in deoxygenating *Prostoecos* haemoglobin much time was given to testing the apparatus. The apparatus was used to determine the dissociation curves of many vertebrate and invertebrate haemoglobins. Of particular interest to the present work on *Prostoecos* haemoglobin are the results of the series of experiments that were conducted on the haemoglobin of *Tubifex* sp.

The haemoglobin from this oligochaete annelid provided the most satisfactory haemoglobin to test the working of the apparatus and the method. This was because *Tubifex* was known to have a low \( P_{50} \) : variously given
as 0.6 mm Hg. (Fox, 1945) and 2.2 mm Hg. (Schaler, 1960). It was readily available in large numbers and the haemoglobin extract could be prepared in a very similar manner to that of Prootoeoes.

**Method**

The haemoglobin was extracted from Tubifex by the method previously described for Prootoeoes (page 221). Of particular interest was the fact that after centrifuging the ground up homogenate at 24,000 r.p.m. (70,000 r.c.f.) for thirty minutes at 3°C ± 1°C it was found that, as for Prootoeoes, the oxyhaemoglobin solution was capped by a light, white precipitate. Excessive contamination by this precipitate was avoided by careful pipetting of the oxyhaemoglobin solution from beneath the surface layer. Any contamination was removed by the same method as was used for Prootoeoes: the oxyhaemoglobin was shaken with a 3:1 mixture of Analar Ether and chloroform followed by centrifuging at approximately 5,000 r.p.m. for five minutes in a bench centrifuge. The fact that this in no way altered the spectral characteristics of the oxyhaemoglobin solution or its ability to combine reversibly with oxygen (see page 255) was taken as further proof that this treatment in no way adversely affected the haemoglobin.

Because of evidence of the activity of microorganisms affecting
the Tubifex haemoglobin during an experiment, the extracted solution was dialysed against a 10 mM solution of sodium fluoride for two days, at 5°C. In later experiments the addition of 10 milligrams of sodium fluoride directly to the haemoglobin was found to prevent any effects of microorganisms.

The purified haemoglobin was then further diluted with phosphate buffer to a convenient concentration. Sufficient of this solution to fill the spectrophotometer cuvette (chamber C) of the tonometer was then pipetted into the apparatus via the side arm of chamber B.

The procedure for determining the dissociation curve of this solution was then carried out, in the manner described previously.

The results of a single experiment are shown in detail to demonstrate the technique (table 2, figs. 42 & 43).

Discussion

The method was found to be very satisfactory for determining the dissociation curve of the high oxygen affinity haemoglobin of Tubifex. The P_{50} was determined in three experiments carried out at pH 7.2 and 20 - 22°C. The values recorded were 0.90, 1.15 and 1.50 mm Hg. All three lie within the value of 0.6 mm determined by Fox (1945) at 17°C and 0 mm CO_{2} and the value of 2.2 mm determined by Sohler (1960) at 19 - 21.5°C and pH 7.1.
Results:

The results of one experiment on the haemoglobin of *Tubifex* sp. are illustrated in Table 21 and figs. 42 & 43.

**Table 21**

**Dissociation Curve of the Haemoglobin of *Tubifex* sp.**

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Barometric pressure (mm Hg)</th>
<th>pH</th>
<th>Volume of tonometer (ml)</th>
<th>Volume of air injected (mls)</th>
<th>Equivalent partial pressure of oxygen (mm Hg)</th>
<th>Absorbance at 546 nm</th>
<th>Percent saturation of haemoglobin</th>
</tr>
</thead>
<tbody>
<tr>
<td>22°C</td>
<td>767.2</td>
<td>7.2</td>
<td>580</td>
<td>0</td>
<td>0</td>
<td>1.37</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>2</td>
<td>0.53</td>
<td>1.46</td>
<td>19.56</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4</td>
<td>1.06</td>
<td>1.535</td>
<td>35.87</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>6</td>
<td>1.59</td>
<td>1.61</td>
<td>52.17</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>8</td>
<td>2.12</td>
<td>1.68</td>
<td>64.40</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10</td>
<td>2.65</td>
<td>1.77</td>
<td>86.95</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12</td>
<td>3.18</td>
<td>1.80</td>
<td>93.50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>14</td>
<td>3.71</td>
<td>1.83</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>16</td>
<td>4.24</td>
<td>1.83</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>157.10</td>
<td>1.83</td>
<td></td>
<td>100</td>
</tr>
</tbody>
</table>
1. Deoxyhaemoglobin - produced by the addition of sodium dithionite.

2. Family of spectra.

Fig. 42. Dissociation curve of Tubifex haemoglobin.
Fig. 43. Dissociation curve of *Tubifex* haemoglobin.
The exact overlap of the spectra in the 'family of spectra' displayed in fig. 42, is a point of importance. As Okazaki and Wittenberg (1965) pointed out, the constancy of the isobestic points throughout the family of spectra, in this case at 505 nm, 530 nm, 555 nm, 574 nm and at 590 nm, demonstrates that there has been no formation of methaemoglobin or other ferric haemoglobin derivatives during the course of the experiment.

It is also interesting to note from fig. 42 that the deoxygenated spectrum, whether obtained by equilibration with oxygen free nitrogen or with sodium dithionite, has a suggestion of a 'shoulder' to the peak (see page 240).

Thus the evidence is that the method can be extremely successful when used for high oxygen affinity haemoglobins such as obtained from Tubifex. Being able to completely deoxygenate a solution of haemoglobin which is known to have a $P_{50}$ in the region of 1 mm Hg. strongly suggests that the method of removing the oxygen from the tonometer and the haemoglobin is successful.

Dissociation curve of haemoglobin extracted from Proctocetes

Method

A dilute solution of haemoglobin was prepared in the manner
previously described. This was pipetted into chamber B of a tonometer which was then evacuated of oxygen in the manner described (page 246).

In an attempt to secure complete deoxygenation of the oxyhaemoglobin of Protoscesas many variations of the basic method were employed. Both methods (see page 246) of the evacuation of the oxygen from the tonometer and its replacement by oxygen free nitrogen were tried. Large scale pressure reductions (of approximately 500 mm Hg.) and subsequent re-entry of oxygen free nitrogen were even carried out in an attempt to deoxygenate the sample. Samples were also left in an atmosphere of oxygen free nitrogen for 24 hours.

Variations in buffers, temperature and the addition of various compounds were tried in the series of experiments. For example, sodium fluoride was added in case microorganisms were affecting the deoxygenation and Bovine serum albumen (B.S.A.) was added to some oxyhaemoglobin solutions to act as a protein buffer. The variations in technique and the results are summarised below for a series of ten experiments that were carried out upon extracted oxyhaemoglobin.

Even if no visible deoxygenation had occurred or if the final spectrum was not characteristic of deoxyhaemoglobin, oxygen was re-admitted and its effect noted.
## Results

### TABLE 22

<table>
<thead>
<tr>
<th>Expt. number</th>
<th>Buffer</th>
<th>pH</th>
<th>Temperature</th>
<th>Addition</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Tris</td>
<td>7.0</td>
<td>22</td>
<td>NaF</td>
<td>small reversible change (fig. 44)</td>
</tr>
<tr>
<td>2</td>
<td>35 mM phosphate</td>
<td>6.9</td>
<td>20</td>
<td>-</td>
<td>small reversible change</td>
</tr>
<tr>
<td>3</td>
<td>Tris</td>
<td>7.0</td>
<td>20</td>
<td>B.s.a. (10 mgm)</td>
<td>small reversible change. No further decxygenation after 24 hours in O₂ free N₂</td>
</tr>
<tr>
<td>4</td>
<td>35 mM phosphate</td>
<td>7.0</td>
<td>20</td>
<td>B.s.a. (10 mgm.)</td>
<td>small reversible change</td>
</tr>
<tr>
<td>5</td>
<td>66 mM phosphate</td>
<td>8.04</td>
<td>20</td>
<td>-</td>
<td>No reversible change</td>
</tr>
<tr>
<td>6</td>
<td>50 mM Borate</td>
<td>7.2</td>
<td>15</td>
<td>-</td>
<td>No reversible change</td>
</tr>
<tr>
<td>7</td>
<td>66 mM phosphate</td>
<td>7.2</td>
<td>5</td>
<td>-</td>
<td>No reversible change (see fig 45B)</td>
</tr>
<tr>
<td>8</td>
<td>66 mM phosphate</td>
<td>7.2</td>
<td>20</td>
<td>-</td>
<td>No reversible change</td>
</tr>
<tr>
<td>9</td>
<td>66 mM phosphate</td>
<td>7.2</td>
<td>5</td>
<td>-</td>
<td>No reversible change</td>
</tr>
<tr>
<td>10</td>
<td>66 mM phosphate</td>
<td>7.2</td>
<td>20</td>
<td>-</td>
<td>Small reversible change (see fig. 45A)</td>
</tr>
</tbody>
</table>
Fig. 44. Spectra (X 5 scale expanded) obtained during attempted dissociation curve of *Proctoeceps* haemoglobin (experiment 1).

- --- Spectrum of oxyhaemoglobin at start.
- ........ Spectrum after 4 hours in O₂ free N₂.
- --- Spectrum after re-admission of air.
- --- Spectrum of deoxyhaemoglobin produced by addition of sodium dithionite.
Fig. 45. Spectra of Proctocecs haemoglobin - Dissociation curve experiments 7 and 10.

A. Experiment 10
1. After nitrogen.
2. After re-admission of air.

B. Experiment 7
1. Before addition of nitrogen.
2. After 6 hours in nitrogen.
Results

At no time was complete deoxygenation followed by reoxygenation accomplished. In many of the early experiments, (which are not included in the table) when the samples of oxyhaemoglobin were somewhat impure and often contaminated with part of the fine, white, fat precipitate, the spectrum was altered after the oxyhaemoglobin had been left in an atmosphere of oxygen free nitrogen for periods up to six hours (see fig. 46). These changes in the spectrum were not reversible and so it is concluded that the spectrum obtained was that of methaemoglobin, or some other ferric derivative, that does not combine reversibly with oxygen.

Davenport (1949a), when attempting to determine the $P_{50}$ of Ascaris perienteric fluid haemoglobin experimentally, noted a similar hastening of the formation of methaemoglobin by exposure to low oxygen tensions and to gentle shaking. As Benesch et al (1964) pointed out, the long exposure of haemoglobin to low oxygen tensions can be undesirable because the rate of oxidation of haemoglobin to methaemoglobin is at a maximum at low oxygen pressure. This is because deoxyhaemoglobin is oxidised very much more rapidly than oxyhaemoglobin.

No denaturation of the oxyhaemoglobin was observed to occur in later experiments when purer solutions of oxyhaemoglobin were used and it is
Fig. 46. Spectrum of a sample of Proctococces haemoglobin after re-admission of air to a sample that had been in an atmosphere of oxygen free nitrogen.
the results of these experiments that are expressed in table 22.

No completely reversible deoxygenation was observed to have occurred in any experiment. In some, for example experiment 1, (see fig. 44), the oxyhaemoglobin spectrum was altered after four hours in oxygen free nitrogen. Upon re-admission of air a small change back towards the double peaked spectrum was observed, but this could not be increased. The spectrum so obtained was not the normal one of oxyhaemoglobin because the position of the peaks was altered from 541 and 579 nm to approximately 538 and 574 nm. There was also the existence of a third peak at approximately 610 nm. Thus it had features in accord with the spectra obtained in some of the early experiments (fig. 46). The addition of sodium dithionite to the solution produced a spectrum of deoxyhaemoglobin for comparison. Thus it can be seen that complete deoxygenation was not achieved and re-admission of air did not cause a reversal back to oxyhaemoglobin.

In other experiments, after long periods in oxygen free nitrogen, the alpha and beta peaks appeared somewhat flattened. This was reversed upon readmission of air (see fig. 45A). During no experiment, although many variations were attempted, could this flattening of the alpha and beta peaks be increased. Further change was not facilitated by further
additions of oxygen free nitrogen, the addition of some impure extract
or even after large scale pressure reductions.

Decoxygenation of the sample of oxyhaemoglobin in the tonometer,
if not denatured, was always brought about at the end of an experiment
by the addition of sodium dithionite.

Discussion

It appears from the complete inability of the author to decoxygenate
the extracted oxyhaemoglobin of Proctoeoes by the use of a technique
that was successfully applied to the high affinity pigment of Tubifex
that the oxyhaemoglobin of Proctoeoes is indeed very resistant to
decoxygenation.

No comparable work has, to the author's knowledge, been carried out
upon the haemoglobin extracted from another trematode. A number of
determinations of oxygen dissociation have been carried out upon
haemoglobins of parasitic nematodes and so any comparison must be made
with the properties of the haemoglobins of the members of this group.

Smith and Gibson (1964) reported that kinetic studies (assuming the
extent of haem – haem reactions was one), indicated that the $P_{50}$ of the
body wall haemoglobin of Ascaris was 0.0108 mm Hg. at 20°C and 0.491 mm Hg.
at 37°C, whereas the $P_{50}$ of the perienteric haemoglobin was 0.00146 mm Hg.
and 0.0112 mm Hg. at 20°C and 37°C respectively.
Okazaki and Wittenberg (1965) determined experimentally that

the P$_{50}$ of *Ascaris* perienteric haemoglobin was $0.001 - 0.0035$ mm Hg. at

pH 7.0 and 20°C. They determined the P$_{50}$ of this haemoglobin using

a tonometer and a method differing somewhat from that used during the

present investigation. The reason that they were able to determine the

P$_{50}$ of a haemoglobin with such a high affinity for oxygen was basically
two fold: *Ascaris* is a large nematode and it is easier to obtain pure

solutions of haemoglobin from the perienteric fluid than it is to obtain

haemoglobin from a small trematode lacking a body fluid. Secondly,
because of the fact that carbon monoxide dissociates from the haemoglobin

of *Ascaris* more rapidly than does oxygen, the deoxygenation of the

haemoglobin was accomplished, by Okazaki and Wittenberg (1965), by

initially converting the oxyhaemoglobin in the tonometer to carbon monoxide

haemoglobin. Once this had been achieved the gas phase in the tonometer

was replaced with nitrogen and the solution was illuminated by a carbon

arc lamp to accelerate the dissociation of the carbon monoxide.

Okazaki and Wittenberg (1965) state that by this method, mainly
deoxyhaemoglobin remained after an hour although it was still

contaminated with some oxy- and ferric haemoglobin. Injections of small

volumes of an oxygen/nitrogen mixture allowed for the determination of the
dissociation curve of this high affinity pigment by a method similar
to that described previously.

Rogers (1949a) found that the haemoglobins of Nematodirus, Haemonchus
and Nippostrongylus also had a high affinity for oxygen. For Nematodirus
and Haemonchus the P 50 was about 0.05 mm Hg. and for Nippostrongylus
was about 3 to 4 times higher.

The suggestion that the extracted oxyhaemoglobin of Proctocces is
extremely resistant to deoxygenation has thus been demonstrated as also
applying to the extracted oxyhaemoglobins of some nematode parasites.

The value of such studies on the dilute oxyhaemoglobin of
invertebrates and vertebrates and the accuracy of the values of P 50
obtained has been questioned. Riggs (1965) reviews the evidence, and
suggests that interpretation of some of the measurements made in the past
may be necessary in the light of the observations that dilution of certain
haemoglobins, such as those of the lamprey (Briehl 1963) and Rana
catesbeiana (Riggs 1965), greatly increases their oxygen affinity. Many
invertebrate haemoglobins have been studied in extremely dilute solutions.
This is true of the studies on Ascaris perianteric haemoglobin by Smith
and Lee (1963) and of the same haemoglobin by Davenport (1949). It must
be true for any haemoglobin that occurs in a small animal at a relatively
low concentration.
This is certainly true for the present study on the haemoglobin of Protozoa. The study on the dissociation curve of the oxyhaemoglobin was conducted on extremely dilute solutions and so the extreme resistance to deoxygenation that was noted may be due, at least in part, to this suggestion.

In vivo observations of the oxyhaemoglobin

If the results obtained from experiments on extracted pigment are open to a certain amount of doubt as regards their significance in vivo then perhaps some information may be gained from observing the reaction of the pigment in the living animal.

Davenport (1949a) found that when Ascaris was deprived of all oxygen, the perienteric fluid haemoglobin was extremely resistant to deoxygenation, but the body wall haemoglobin could be deoxygenated very slowly by the nematode: the activity of the nematode ceased when this haemoglobin was deoxygenated. Davenport (1949 a & b) found that the inability of Ascaris to deoxygenate the perienteric fluid haemoglobin was similarly found in Strongylus. After three hours in vacuo at 20°C, there was little or no visible deoxygenation of these haemoglobins.

A deoxygenation of the oxyhaemoglobin of Callamus in vivo, was observed by Sharton (1941) when the nematode was placed under anaerobic...
conditions and it became reoxygenated upon re-admission of air. Von Brand (1937) observed that the haemoglobin of *Eustrongylloides* larvae became deoxygenated under anaerobic conditions and Van Grombergen (1954) observed a similar effect with *Heterakis*.

Rogers (1949 a & b), who demonstrated that the extracted oxyhaemoglobins of *Nematodirus*, *Haemonchus* and *Hippobosporhylus* were very resistant to deoxygenation (see p267) found that when the oxygen tension in the medium surrounding the parasites at 37°C fell below 13 mm (for *Hippobosporhylus*) or 9 mm (for *Haemonchus* and *Nematodirus*) the oxyhaemoglobin became deoxygenated.

Similarly, it is interesting to note that Palmer and Chapman (1970) demonstrated that the haemoglobin of *Tubifex*, in vivo, became deoxygenated below 11.4 mm Hg partial pressure of O₂. This is to be compared to the P 50 for the extracted pigment which is below 2 mm Hg. (see page 253).

Thus the deoxygenation of the pigment, in vivo, appears in some cases, to occur at a higher partial pressure of oxygen than for the extracted pigment. However, there is no evidence that the partial pressure of oxygen inside the animal is the same as that in the external medium.

Because of the necessity of diffusion across the body wall and through the,
possibly, relatively impermeable cuticle, and even if accelerated by
the haemoglobin, the oxygen tension within these nematodes and Tubifex
may be much lower than that of the external medium. In this way these
animals would be similar to the Crayfish, where the P O₂ in the
haemolymph is only 2 - 4 mm Hg, even when the animal is exposed to an
external oxygen tension of approximately 150 mm Hg. (Larimer 1964).

Thus knowledge of the external partial pressure of oxygen does not
help to show at what partial pressure the pigment is actually half
saturated (P 50) in the living animal and so no comparison can be made
with the values of P 50 obtained from experiments on the extracted pigment.
These experiments are of value in demonstrating under what, if any,
conditions of oxygen tension the pigment within the animal could be
functional as an oxygen carrier or store.

Reactions of the haemoglobin of Proctoeces in vivo

Method

An airtight chamber was constructed on a microscope slide by sealing
a brass washer, with a 6 mm diameter hole, onto the slide with silicone
grease. Into the well were placed between ten and twenty Proctoeces and
the rest of the well was filled with pasteurised sea water. The top of
the washer was well greased with silicone grease and a coverslip was applied
to it. Care was taken to ensure that the seal was complete. The slide was placed on the stage of a microscope, the eye-piece of which had been replaced by a microspectroscope. The alpha and beta absorption bands of the haemoglobin were clearly visible as dark bands on the spectrum viewed through the microspectroscope. In between the examinations for any change in spectrum the slide was kept at a constant temperature.

Five such experiments were carried out.

Results

At no time during any of the five experiments did the alpha and beta bands alter in position or disappear. In one experiment carried out at 20°C ± 1°C, eight out of ten Protozoa were still alive after 38 hours sealed on the slide. No change in the bands of absorbance were detectable after this period, although at a respiration rate of 0.168 µl/mg. fresh wt./hour (Freeman 1962b) the ten Protozoa would have consumed all the oxygen from the sea water within the first hour.

The ability of Protozoa to survive under anaerobic conditions is illustrated by the results of another experiment. Ten Protozoa were placed in pasteurized sea water in a small vessel. Oxygen free nitrogen was bubbled through the sea water for four hours before the vessel was sealed; care being taken that the atmosphere above the sea water was oxygen
free nitrogen. After nineteen days at 15°C, 50% of the ten Proctoeces were still alive.

Discussion

It appears that not only is it impossible to deoxygenate dilute solutions of the extracted pigment but also that the trematode is unable to deoxygenate the pigment when kept under anaerobic conditions. In this respect it appears to be similar to the perienteric fluid of Ascaris and Strongylus (Davenport 1949a & b).

Possible functions of the haemoglobin of Proctoeces

Where haemoglobin occurs in a circulatory system its function is usually supposed to be basically one of transporting oxygen from an area of high oxygen tension (e.g. the gills) to areas of lower oxygen tensions (the tissues). For the haemoglobin to function in this manner the oxygen tensions at which the pigment loads and unloads its oxygen must be related to the tensions occurring at the two sites. Among the lower vertebrates and invertebrates the function and the importance of haemoglobin varies enormously. The fact that in Proctoeces the haemoglobin is contained within tissue and appears refractory to deoxygenation precludes this basic function. Related oxygen carrying functions have been suggested in the literature for other haemoglobins and these can be considered under the headings of oxygen storage and facilitated diffusion.
It has been demonstrated that the oxygen tension within the kidney of *S. plana* can fall to below 1 mm Hg during a period of tidal exposure (see page 196) but the experimental evidence is that the haemoglobin of *Protoecae* would not yield its oxygen, even under such conditions. This latter point would suggest that the pigment does not act as an oxygen store for *Protoecae* for periods of low oxygen tension.

This point was discussed by Freeman (1963) who concluded that on the basis of the known respiratory rate of *Protoecae* (Freeman 1962b) and upon the assumption that the haemoglobin content of *Protoecae* was of the same order as that determined by Coel (1959) for *Gastrothylax crumenifer*, the haemoglobin would store enough oxygen to maintain a normal aerobic metabolism at 5°C for about 25 minutes. Coel has since stated that his figure for the haemoglobin content of *Gastrothylax crumenifer* was ten times too high (see Lee and Smith 1965) and so the time should be 2.5 minutes. The possibility that the haemoglobin of *Protoecae* functions as an oxygen store appears unlikely.

Freeman (1963) suggested that if it was at all possible that the haemoglobin of *Protoecae* could give up its oxygen to cellular metabolic processes its function may perhaps be to facilitate oxygen diffusion.

The phenomenon of haemoglobin - facilitated diffusion of oxygen
was discovered independently by Wittenberg (1959) and Scholander (1960) and has since been investigated by many workers including Hämmonson (1963). This latter author (1962) also demonstrated that facilitated oxygen diffusion could occur across membranes even at partial pressures high enough to fully saturate the haemoglobin.

Wittenberg (1965) suggested that the haemoglobin of some nematode parasites that live embedded in the walls of the intestine may facilitate oxygen diffusion. This was partly based upon the calculations of Rogers (1949b) who demonstrated that the measured rate of oxygen uptake was several times greater than the rate calculated for free diffusion of oxygen. Wittenberg (1965) inferred that the nematode haemoglobin must in some way facilitate the entry of oxygen into the animal.

Wittenberg (1966) elucidated the molecular mechanism of haemoglobin facilitated diffusion and concluded that it required no special property of haemoglobin other than reversible oxygen binding. The fact that this condition does not appear to be fulfilled by the haemoglobin of Prostoecon suggests that facilitated diffusion is a function that cannot be assigned to this pigment.

The more usual oxygen-carrying role of haemoglobin, variations of which have been discussed, does not appear to apply to the haemoglobin of
Prootooeos. This is further suggested by the observations of Freeman (1963) and of the present author upon the survival of Prootooeos under anaerobic conditions. Freeman (1963) demonstrated that 50% of a sample of 100 Prootooeos were able to survive at 5°C for 21 days under an atmosphere of nitrogen. Similarly, as described previously (page 271), the present author noted that 50% of a sample of ten Prootooeos kept under an atmosphere of oxygen free nitrogen were still alive after nineteen days.

This prolonged survival under anaerobic conditions further suggests that the haemoglobin of Prootooeos cannot function significantly as an oxygen store or be necessary as an oxygen transport system. In either case it cannot be a limiting factor for the observed period of this anaerobic survival.

If the more usual oxygen carrying function of haemoglobin is to be discounted then other possible reasons for its occurrence in Prootooeos must be sought.

Jones (1963) discusses the suggestion of Manwell (1959) that haemoglobin, especially in invertebrate animals, may act as an oxygen buffer. It is known that many metabolic processes are sensitive to oxygen tension and thus it has been suggested that the function of some haemoglobins may be to regulate the oxygen tensions in the cells within tolerable limits. This suggestion of Manwell (1959) would be difficult
to prove and would seem to be irrelevant for *Protozoa* but it does present a novel suggestion for the function of some haemoglobins.

Smith and Lee (1963) have suggested that the function of the perienteric haemoglobin of *Ascaris* may be as a source of haematin for the eggs and a source from which other haemoproteins are made.

It has been suggested that haemoglobin may have an enzymatic function where it occurs in such organisms as root nodules, *Protozoa* and yeasts. The evidence for the suggestion that haemoglobin in root nodules may be involved in nitrogen fixation has been extensively reviewed by Riggs (1965) who concludes that its exact function remains unclear.

This latter statement also applies to the haemoglobins of nematode and trematode parasites. More work is needed on the haemoglobins of both of these groups of parasites to provide experimental evidence as to the function of the haemoglobin. Many of these high affinity haemoglobins may facilitate the diffusion of oxygen from the environment to the tissues as suggested by Wittenberg (1965). A comparison of the oxygen tensions at which the extracted haemoglobin is half saturated with the environmental oxygen tensions may be misleading as the extent of the diffusion barrier presented by the body wall and cuticle is unknown. It may be that the function of the haemoglobin, especially when it occurs in the body wall,
is to help to overcome this barrier.

Unfortunately, because of the fact that the haemoglobin of Proctoeoes, which is distributed throughout the tissues, is resistant to deoxygenation, it is unlikely that it can function as an oxygen storage or an oxygen transport system. Other functions that have been suggested for haemoglobins also appear unlikely although equally unacceptable would be the suggestion that it represents a functionless by-product of the worm's metabolism.

Much has still to be learnt of the function of the high affinity haemoglobins of many invertebrates. Its occurrence in a parasite of an invertebrate lacking the pigment presents problems of synthesis and function. Nothing is known of the former problem and at the present, even after this investigation, the function of the haemoglobin of Proctoeoes must remain a mystery.
CHAPTER X

THE ENVIRONMENT OF PROCTOECES

It is interesting, in the light of the results, to consider the possible conditions in the kidney of S. plana.

It has been shown that the tubular fluid in which the parasite lives is isosmotic with the external medium and with the blood over a wide range (see page 145). Although this is true, the small extent of the salinity variations in the vicinity of Southend, on the north coast of the Thames Estuary, would most probably mean that Proctoeces is never exposed to a sea water dilution of below 75% (see page 157). Even if it was exposed to extensive variations it would be able to survive for long periods without being able to regulate (see pages 153-156).

Although no complete analyses have, to the author's knowledge, been carried out upon the constituents of the kidney fluid of marine lamellibranchs, it is constructive to consider its possible composition in relation to the blood, with which it is isosmotic.

Analyses of the blood of the lamellibranchs Mya arenaria, Pecten maximus and Ensis ensis (Robertson, 1949) and Mytilus edulis (Potts 1954) have demonstrated that the haemolymph of these animals resembles sea water in total concentration and composition with the exception that it contains
slightly higher concentrations of Ca$^{++}$ and K$^+$. 

In molluscs filtration of the haemolymph seems to take place through the wall of the heart into the surrounding pericardium (Potts and Parry 1964) but little information is available as to what happens to the ions in this fluid once it is in the pericardium. Robertson (1949) did analyse the pericardial fluid of Pisaster and found that the chloride and sulphate concentrations were identical with those of the blood. Lange (1963) showed that the chloride in the pericardial fluid of Hytilus edulis was the same as that in sea water.

The fluid, together with the secretion of the pericardial glands, then passes from the pericardium and into the excretory tubules. Modification of the filtrate occurs in the tubule, mainly by the addition of nitrogenous excretory compounds, of which urea and the amino acids taurine and creatine have been identified in twenty two lamellibranchs (Delaunay 1931). The final ionic composition of this fluid in the kidney tubules will depend on how far such processes as secretion and resorption of ions take place in the epithelium of the tubule. To the author's knowledge, no investigations have been carried out to demonstrate the extent of such processes in estuarine lamellibranchs.

Because of the similarity of the blood of lamellibranchs with sea
water and because of the fact that little modification of ions appears
to occur once this has been filtered through the heart wall, it seems
probable that the electrolytes present in the kidney fluid will be
extremely similar to those in the blood and thus to those in the bathing
sea water. Certain ions may differ in concentration and organic
compounds such as amino acids and possibly carbohydrates may be present.

Thus it appears that Proctoeces is essentially living in a fluid that
is extremely similar to the bathing sea water, both in total
concentration and in composition. If this is so then the prolonged
survival of adult Proctoeces in sea water is readily understandable (see
page 156). Thus, although the most prolonged survival was found to occur
at 30 and 50% sea water, 50% of the Proctoeces in 100% sea water at
6°C ± 1°C were still alive after 180 hours (7.5 days) and the last died
only after 300 hours (12.5 days). It is possible that the absence of
organic food substances in the various dilutions of pasteurised sea water
was a factor contributing to the parasites' ultimate death.

The slightly higher concentration of Ca++ that is likely to be
present in the kidney fluid may be in part, the reason for the
occurrence of "fossilised" Proctoeces. The occurrence of these dead, but
preserved, parasites within the kidney of S. plana was first noted by
Freeman and Llewellyn (1958). They observed that they varied in colour from light to dark brown and that the darker ones were very hard. These authors found that approximately 10% of the Sorobicularia at Chalkwell were infected with these dead Protoeces with as many as six in a single host.

These dead Protoeces were evident in S. plana examined during the present investigation. The percentage of S. plana from Clifftown, that were so infected, varied (see fig. 47) and the maximum number recorded from a single host was four. From the graph (fig. 47) it is evident that the incidence of dead parasites increased over the period of study pari passu with the overall increase in the infection level of the Sorobicularia (see page 60).

Dead Protoeces were always found together with living trematodes in the same host but their significance is obscure. Von Brand (1952) states that some nematode parasites become calcified after death and a similar phenomenon has been described for a trematode by Macfarlane (1939).

Lamellibranchs will calcify any intruder between the mantle and the shell and so form pearls. The metacercarial stages of Gymnophallus margaritatum often become calcified in lamellibranchs and hence are often termed the "pearl trematodes" (Jameson 1902). Pearls are often evident in
Fig. 47. The percentage of *S. plana* from Clifftown (Site 7) containing dead *P. subtenuis*. 

![Graph showing the percentage of dead *P. subtenuis* in *S. plana* samples from Clifftown (Site 7) over the years 1968 to 1970. The x-axis represents the months from January to December, and the y-axis represents the percentage of infected *S. plana*. The graph shows a trend of increasing infection from 1968 to 1970.]
S. plana but it is not known whether they are caused by trematode larvae or other irritant matter.

Because the body fluids of lamellibranchs are saturated with the aragonite form of calcium carbonate (Potts and Parry, 1964) it is perhaps not so surprising that dead Protozoa in the kidney do become calcified. Perhaps what is more surprising is that once dead a trematode is not immediately broken down by bacteria. This suggests the kidney is sterile.

Although the kidney fluid may be similar to that of the external sea water in total concentration and composition, the oxygen content of the fluid has been shown to be always less than that of the bathing medium; in the pumping animal the oxygen tension in the kidney was approximately 50% saturated with respect to the oxygen tension in the bathing sea water, and during an emersion in excess of three hours the oxygen tension has been shown to fall to levels approaching zero.

The presence of haemoglobin does not appear to assist Protozoa during its sustained survival under anaerobic conditions. The fact that the haemoglobin of Protozoa does not appear to liberate its oxygen, either in vivo or in extracted solution, suggests that oxygen transport, oxygen storage or facilitated diffusion functions cannot be suggested for this pigment.
Some aspects of the environment of Proctoeca, and its reactions to the conditions, have been elucidated by this research work although many problems still exist. The nature of the food of the parasite and its ability to synthesise haemoglobin whilst in a host lacking the pigment, are two physiological problems that would warrant investigation.
SUMMARY

SECTION I

1. *Protooea subtenuis* was only found within the kidney of

    *Scolobicularia plana* collected from locations on the north coast
    of the Thames Estuary.

2. The pattern of abundance of *Protooea* was distinct and was

    repeated in each of the three years of study.

3. At Clifftown the parasite was very successful; Over 95% of all

    *S. plana* collected were infected. An increase in the level of

    infection occurred over the period of study to a level at which

    there was an average of 4–5 *Protooea* per host in 1969/70.

4. A maximum of fourteen *Protooea* were found in a single host.

    The larger/older the *S. plana* the greater was the number of

    *Protooea* that they contained.

5. The pattern of distribution of *U. edulis* was very similar to that

    of *Protooea*.

6. Sporocysts resembling those described for members of the genus

    *Protooea* by American workers were found from *U. edulis* on two

    occasions.

7. A life cycle has been suggested and some general points considered.
SECTION II

8. The kidney fluid of *S. plana* was found to be isosmotic with respect to the medium from a depression of freezing-point of $0.478^\circ C$ to $1.695^\circ C$.

9. Proctoeoes was able to survive for significant periods in sea water dilutions of 20% to 100%. The greatest duration of survival being in 30% and 50% sea water.

10. Proctoeoes was found to be isosmotic with respect to the bathing medium from a depression of freezing-point of $0.585^\circ C$ to $2.02^\circ C$.

11. The polarograph electrode used in the investigation of oxygen tensions within the kidney of *S. plana* was found to have a linear calibration with respect to oxygen tension in sea water, and to temperature.

12. When immersed the oxygen tension within the kidney of *S. plana* was approximately 70 mm Hg. and fell to a level approaching zero upon emersion of the animal.

13. The determination of the respiration rate and the mantle cavity volume of *S. plana* demonstrated that it would be unable to obtain sufficient oxygen to maintain its aerobic respiration rate during periods of emersion.
14. The peaks of absorbance of the oxyhaemoglobin of Proctoeces were found to be at 579, 541 and 417 nm. The absorbance of the beta peak was greater than that of the alpha peak.

15. The peaks of absorbance of the deoxyhaemoglobin were at 566, 550 and 429 nm, there being a double peak in the visible region.

16. It was not possible to deoxygenate the haemoglobin of Proctoeces using a method similar to that found to be successful for determining the dissociation curve of the high affinity pigment of Tubifex sp.

17. In vivo observations of the haemoglobin of Proctoeces demonstrated that no change in the spectrum occurred when the animal was kept under anaerobic conditions.

18. Proctoeces was found to survive under anaerobic conditions for substantial periods.

19. The presence of dead, but preserved, Proctoeces in the kidney of S. plana could possibly be related to the fact that the blood of lamellibranchs is saturated with the aragonite form of calcium carbonate.
REFERENCES
14. The peaks of absorbance of the oxyhaemoglobin of Prootoeooe were found to be at 579, 541 and 417 nm. The absorbance of the beta peak was greater than that of the alpha peak.

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18. Prootoeooe was found to survive under anaerobic conditions for substantial periods.

19. The presence of dead, but preserved, Prootoeooe in the kidney of S. plana could possibly be related to the fact that the blood of lamellibranchs is saturated with the aragonite form of calcium carbonate.
REFERENCES
289.


Science, 144, 68 - 69.


BOYDEN, C.R. 1970. Comparative studies on Cerastoderma edule (L) and C. glaucum (Poiret). 
Ph. D. Thesis, Queen Mary College, University of London.


J. Parasit., 23, 225.


BRAND, T. VON. 1952. Chemical Physiology of Endoparasitic animals. 


BRIEHL, R.W. 1963. The relation between the oxygen equilibrium and aggregation of sub-units in Lamprey haemoglobin. 

J. gen. Physiol. 33, 475 - 495.

CABLE, R.M. 1953. Life cycle of Parvatrema boringuenae and systematic position of the sub-family Gymnopallinae.
J. Parasit., 39, 408 - 421.


COLE, H.A. 1935. On some larval trematode parasites of the mussel (Mytilus edulis) and the Cockle (Cardium edule).
Parasitology, 27, 276 - 280.

COLE, W. H. 1940. Composition of fluids and sera of some marine animals, and of the sea water in which they live.

Measurements of oxygen tension in the lumen of the small intestine of domestic ducks.


DELANEAY, R. 1931. Excretion azotee des invertébres.
Biol. Rev., 6, 265 - 301.

J. clin. Path. 16, 183.


DOLJUS, R., Ph. 1924. Polynéa et progrénese de la larve metacerctaire de Pleurogenes medianus.
DOLLFUS, R., Ph. 1965. Metacercariae Proctoeces progeneticus
(Trematoda, Digenea) chez une Gibbula (Gastropoda, Prosobranchiata)
de la Côte Atlantique du Maroc. Observations sur la famille
Fellodistomatidae.
Annals. de Parasitologie (Paris) 39, 755 - 774.

Recherche sur le parasitisme des mollusques bivalves de la côte
portugaise.

solutes of high molecular weight.

FORRES, E., and HANLEY, S. 1853. A history of British Mollusca and
their shells. London, Jan Van Voorst.

FOX, C.J.J. 1907. On the coefficients of absorption of the atmospheric
gases in distilled water and sea water. I. Nitrogen and oxygen.

J. exp. Biol. 21, 161 - 164.

FREEMAN, R.F.H. 1957. Paravortok scoobiculariae (Graaf) in Great

FREEMAN, R.F.H. 1962 a. Experimental infection of two species of
Nerites with the digenean Proctoeces subtenuis.

FREEMAN, R.F.H. 1962 b. Volumetric respirometer measurements of the
oxygen consumption of the digenetic trematode Proctoeces subtenuis.

FREEMAN, R.F.H. 1963. Haemoglobin in the digenetic trematode


Ray Society publication.


Z. Parasitenk. 19, 362 - 363.


Parasitology, 56, 101 - 104.


Enzymologia 13, 241 - 257.


HERDAN, W.A. 1893. Report on investigations carried out in 1892 in connection with the Lancashire sea-fisheries laboratory at University College, Liverpool.


KANESHIRA, E.S., HOLZ, C.G., DUNHAM, P.B. 1969b.

II. Regulation on intracellular free amino acids. Ibid., 137, 161 - 169.


MACKENZIE, K., and GIBSON, D. 1970. Ecological studies of some parasites of the Plaice, Pleuronectes platessa (L), and the Flounder, Pleuronectes flesus (L).

Aspects of fish parasitology. Symposia of British Soc. of Parasitology, Vol. 8, 1 - 42.


MOORE, H.B. 1931. Muds of Clyde Sea area. III. Chemical and physical conditions: rate and nature of sedimentation and fauna.


PALMER, M.F., and CHAPMAN, G. 1970. The state of oxygenation of haemoglobin in the blood of living Tubifex.


Rogers, W.P. 1949a. II. The properties of the haemoglobins as studied in living parasites. Ibid., 399 - 407.


SOOTT, A. 1895. Report on the investigations carried on in 1894 in connection with the Lancashire Sea Fisheries Laboratory at University College, Liverpool.


STEPHENSON, W. 1947b. III. Egg shell formation. Ibid., 128 - 139.


THORPE, N. 1970. Comparative study of tidal rhythmicity in
Sorobicularia plana (da Costa) and Macoma balthica (L). J. Exp.

UZMANN, J.R. 1953. Cercaria milfordensis — microercous trematode larva
from Mytilus edulis and its effect on the host. J. Parasit.,

VAN DAM, L. 1935. On the utilisation of oxygen by Mya arenaria.
J. exp. Biol., 12, 86 — 94.

WEIL, E., and PANTIN, C.F.A. 1931. The adaptation of Gundus ulvae II.

WHARTON, G.W. 1941. The function of respiratory pigments of certain

WHEELER, A. 1969. The fishes of the British Isles and North West
Europe. Macmillan.

canal of fish as an environment for helminth parasites. Aspects
of fish parasitology. Symposia of British Society of Parasitology,
Vol. 8, 43 — 47.

Haemoglobins of invertebrate tissues. Nerve haemoglobins of

A novel reaction of haemoglobin in invertebrate tissues. II.

WITTEMBERG, J.B. 1959. Oxygen transport: a new function proposed for

J. gen. Physiol., 49, 57 — 74.

WITTEMBERG, J.B. 1966. The molecular mechanism of haemoglobin


ADDENDUM TO REFERENCES


Cambridge University Press.

